Combination of cell immortalization and apoptosis induction to engineer decellularized matrices as bone graft materials

Inauguraldissertation
zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
Paul Bourgine
aus Le Trait, France

Basel, Schweiz, 2013

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Basel, den 17.09.2013
(Datum der Genehmigung durch die Fakultät)

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PhD Studies in Medical and Biological Research - Faculty of Science

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September 2013

“Our revels now are ended. These our actors,
As I foretold you, were all spirits, and
Are melted into air, into thin air:
And like the baseless fabric of this vision,
The cloud-capp'd tow'rs, the gorgeous palaces,
The solemn temples, the great globe itself,
Yea, all which it inherit, shall dissolve,
And, like this insubstantial pageant faded,
Leave not a rack behind. We are such stuff
As dreams are made on; and our little life
Is rounded with a sleep.”

The Tempest, William Shakespeare

Acknowledgements

I warmly thank all my friends and colleagues for the continuous support. I would like to lay down those few words for my family.

Merci papa, merci maman pour tout ce que vous m'avez apporté et ce que vous continuerez à me procurer. Je vous aime et ce pour encore quelques années. Pareil pour les frangins.

P.S: J'aurais juste souhaité moins de coups de chaussons parce que je n'étais quand même pas si turbulent ou alors c'était à cause de mes frères qui avaient commencés.

Abstract

Infection, trauma or tumors can generate critical bone defects in which the regeneration is compromised, thus necessitating the development of suitable repair strategies. Conventional tissue-engineered approaches proposed the use of a three-dimensional (3D) scaffold that supports cell growth and differentiation, in which the seeded progenitor cells can secrete an extra-cellular matrix (ECM) coating the material. The resulting bone graft can then be implanted in the patient in an autologous set-up. However, a more attractive paradigm consists in the removal of the cellular fraction from the graft prior to its implantation in order to avoid immuno-matching requirements, toward a universal exploitation of the graft. This conceptual strategy relies on the capacity of osteoinductive signals embedded in the ECM to instruct endogenous cells toward bone repair.

The success of this approach requires a standard cell source capable of secreting an osteo-inductive ECM, but also the development of a suitable decellularization protocol that can lead to both an efficient cell removal from the graft and the preservation of ECM properties.

In this thesis, I report the combined development of a standardized cell source and an apoptotic-based decellularization strategy, through the generation of a death-inducible human Mesenchymal Stromal Cell (hMSC) line. This cell line was shown to retain typical hMSC properties while continuously proliferating without signs of tumorigenicity, and being efficiently inducible toward apoptosis. Using this unlimited and well-characterized cellular tool, we successfully generated acellular ECM-coated graft within a 3D perfusion bioreactor. In particular, the induction of cell apoptosis was used as a decellularization procedure leading to the better preservation of key ECM components, as compared to the conventional approach.

Overall, by relying on both a novel cell source and a new decellularization approach, the developed protocol may lead to the development of a bone repair treatment with superior standardization and possibly cost-effectiveness as compared to current strategies.

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Chapter 1

Introduction

1 Bone

1.1 Structure and function

Bone is a rigid but porous organ involved in structural, metabolic but also physiological processes in the body. Together with its living compartment, the particular structure of bones leads to a unique fulfillment of functions.

Bone extracellular matrix (ECM) is of mixed composition, including an inorganic (calcium hydroxyapatite crystals) and an organic part mainly composed of collagen type 1, but also of key proteins such as osteocalcin, osteonectin, bone sialo protein and osteopontin (legeros1). The bone ECM displays a relatively low elasticity but essential rigidity that confers the strong robustness to the structure. The solidity of bones has to be correlated with their mechanical function, bones serving both as template for body support and as natural protective barrier. In addition, together with the muscles they participate to the body movement.

The bone matrix is a very dynamic environment orchestrated by its living fraction. Bone cells include osteoblast, osteoclast and osteocyte that are respectively responsible for bone mineralization, resorption and tissue homeostasis (2). This bone remodeling process is essential as bones serve as ions reservoir, balancing the body mineral content (e.g. Ca^{2+} turnover).

Bones are organized in two distinctive structures: the peripheral cortical bone and the internal trabecular bone. The cortical part is compact whereas the trabecular one is spongy and surrounded by the marrow compartment that fills the cavity (figure 1). The bone marrow is a tissue mainly composed of blood, fat and fibrotic cells. Importantly, long bones are the hematopoiesis center, a process giving birth to the entire white (myeloid and lymphoid cells) and red cells (erythrocytes and megakaryocytes) population.

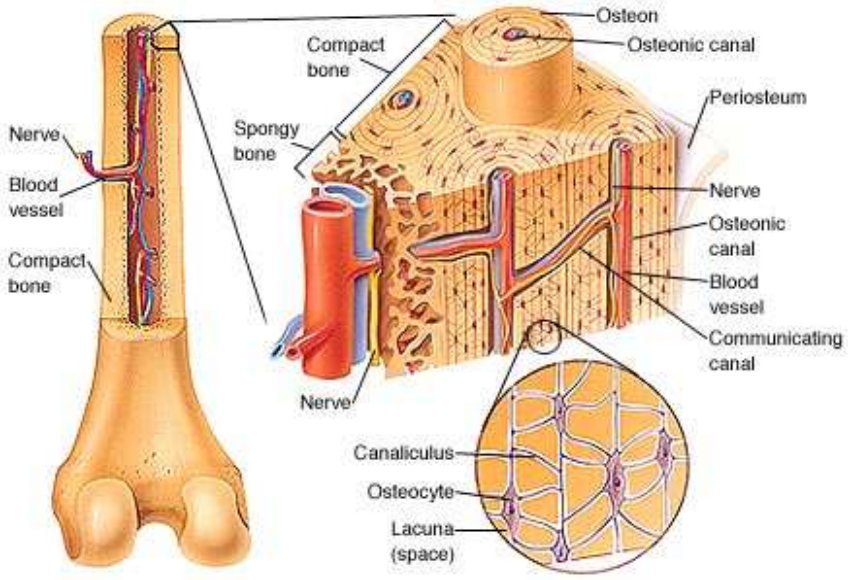

Figure 1. Structure of bone.
(http://www.mhhe.com/biosci/esp/2001_gbio/folder_structure/an/m5/s2/index.htm)

1.2 Bone healing & clinical need

Bone is an important organ and its integrity is crucial for the proper protection, locomotion and functioning of the body. Upon trauma, bones can naturally heal by themselves notably through the presence of osteoprogenitors driving the restoration of the tissue (3) with identical physical properties than the original one. However, infections, tumors or accidents can result in bone defects with a compromised regeneration. Healing problems can be associated with a poorly vascularised environment, a distance over 2 cm between bone ends or the non-stabilization of the fracture-healing site (4). Overall, with 2.2 million bone grafts are performed yearly (5), the healing of bone represents a major clinical concern making the development of bone regeneration therapy a necessity.

1.3 Existing bone repair strategies

The repair of bone defects can be treated via a variety of clinical approaches. The current clinical gold standard consists in the transplantation of autologous bone graft (e.g. extracted from the iliac crest or the fibula) used as osteogenic substitute. Despite the suitability of this approach, it is associated with intrinsic harvest site morbidity and the limited availability of the material (6, 7). The use of

allograft or xenografts can be considered as potential alternatives but their immunogenicity, combined with the risk of disease transmission still raises concerns (8, 9).

Synthetic scaffolds (e.g. titanium, ceramic) allow avoiding immunogenic issues and can successfully provide a structural template. Nevertheless, these bone substitution materials are not necessarily osteoinductive/osteogenic enough and problems in regards to their biocompatibility and/or biodegradability may arise from (10).

Another possibility relies on the injection of growth factors such as human bone morphogenetic proteins (BMPs), use as strong inducer of osteogenesis. Despite a demonstrated bone formation efficiency, the required high doses are associated with aberrant bone formation (11, 12), neurotoxicity and cancer development, leading to a difficult tuning for safe local application (12).

Taken together, the aforementioned strategies suffer from clear limitations and may not be sufficient to promote a fast, complete and stable bone repair.

1.4 Tissue engineering approaches

Progress made in the fields of stem cell biology and bioengineering lead to the development of *in vitro* engineered tissue. The approach consists in the use of a synthetic or natural three-dimensional (3D) scaffold that supports cell growth and differentiation, in which the patient seeded progenitor cells can secrete an ECM coating the material (figure 2A). The resulting bone graft contains both living osteoprogenitors that participate to the new bone formation following implantation, but also osteoinductive signals embedded in the ECM that can instruct endogenous cells toward bone repair (13-15). Limits arising from this approach come from the cell source that displays an intrinsic donor-to donor variation and is of limited supply. Moreover, this strategy only leads to the autologous exploitation of the generated bone substitute.

Therefore, a more attractive paradigm has been proposed through the *in vitro* generation of bone grafts followed by their decellularization prior to implantation into a defect site (figure 2B). The hypothesis lying behind this conceptual approach is that the remaining ECM can induce bone regeneration despite the cellular depletion, through the preservation of key instructive factors

entrapped in the tissue. Importantly, the resulting acellular ECM-coated material may not be immunogenic as the ECM proteins are well conserved even across species (16). The absence of immuno-matching requirements may lead to the generation of universal, non-patient specific graft.

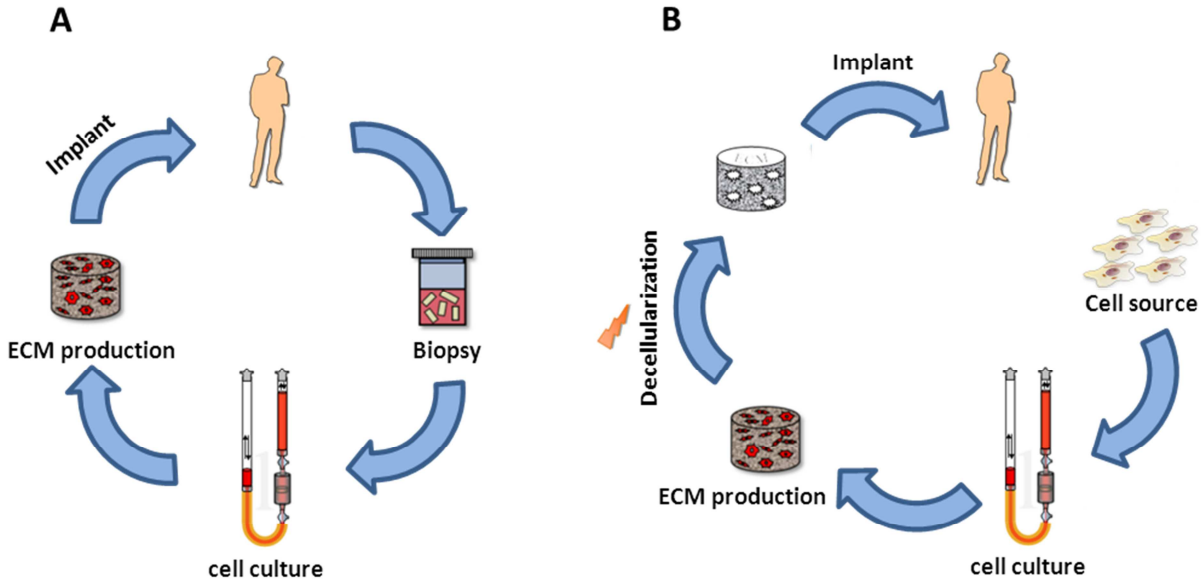

Figure 2. Tissue Engineering approaches. Starting from a biopsy, a graft can be engineered *in vitro* and re-implanted into the defect site (A). An alternative concept (B) aims at decellularizing the generated graft prior to implantation. The acellular graft can then be implanted in any patients to promote an efficient bone repair.

From a clinical standpoint, decellularized ECM with an intrinsic capacity to induce bone formation would be highly attractive. In particular, it would avoid autologous cell processing, which is time consuming and costly, but also lead to a standardized product as by-passing inter-individual variability that severely limits current clinical applications. Overall, this strategy may facilitate the clinical translation of the produced graft, as more cost-effective, feasible and possibly effective than conventional tissue engineering strategies.

2 Critical components of conventional engineered bone substitutes

The success of tissue engineering based strategies may highly depend on key parameters such as the cell source, the scaffolding material, the culture conditions, the decellularization strategy, but also of the vascularization post-implantation, providing direct evidence of successful engraftment.

2.1 Cell source

The engineering of biological bone graft involved the seeding of osteogenic cells on 3D scaffolding material. Different cell sources have been already used for the generation of osteogenic graft (figure 3) (17), such as adipose (18), muscle (19), cord blood (20), and more recently embryonic (21) or induced pluripotent stem cells (22). Nevertheless, bone marrow-derived mesenchymal stromal cells (BMSC) remains the most common cell source for tissue engineering, from both an experimental and clinical point of view (23).

BMSC are an easily accessible cell source that can be isolated from bone-marrow aspirate through a minimally invasive procedure. BMSC are defined as a subset of non-hematopoietic, multipotent and self-renewing stromal cells (24). Those cells are isolated on the basis of their adherence and colony-forming unit-fibroblasts (CFU-F) capacity following whole bone-marrow plating. BMSC are currently described as positive for the CD105, CD73, CD90 markers and negative for CD34, CD14/11b, CD79a/19 and HLA-DR surface molecules (Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy). However, a complete panel of surface markers defining these cells still needs to be identified.

BMSC are promising candidates for regenerative purposes because of their demonstrated multi-lineage differentiation capacity. BMSC have been shown to be able to differentiate into the three main lineages of mesenchymal tissue, namely bone, cartilage and adipose (25). BMSC are also described as being capable to migrate and engraft at injury sites (26) triggering immuno-modulation properties and eventually generating a local immuno-suppressive effect (27). This can potentially lead to an allogeneic exploitation of these cells (28-30), as currently investigated in the context of bone marrow transplant (31).

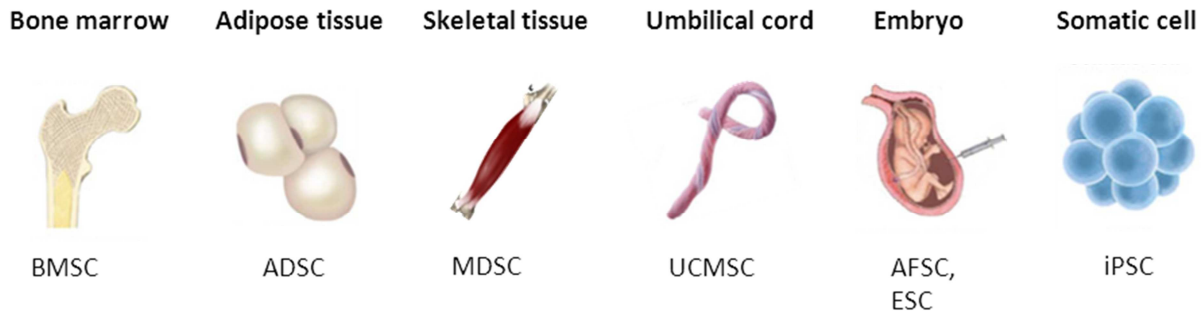

Figure 3. Cell source for bone tissue engineering. BMSC; Bone marrow-derived mesenchymal stromal cells, ADSC; Adipose-derived mesenchymal stromal cells, MDSC; Muscle-derived mesenchymal stromal cells, UCMSC; Umbilical cord-derived mesenchymal stromal cells, AFSC; Amniotic fluid-derived mesenchymal stromal cells, ESC; Embryonic stem cells, iPS; induced pluripotent stem cells. *Adapted from (17).*

Nevertheless, the inherent problems common to all cell sources -including BMSC, arise from the limited availability and the variability among donors (32). In fact, the low amount of osteoprogenitor cells isolated from a biopsy typically mandates an *in vitro* expansion phase. In particular, the frequency of BMSC within bone marrow aspirates does not exceed 0.001 to 0.1% of nucleated cells and their amplification is limited by the replicative senescence phenomenon occurring in *in vitro* culture after a certain number of population doublings (PD) (33, 34). Moreover, the differentiation potential of BMSC -and subsequently their capacity to generate a mature tissue, is reduced along with their population doubling level (32, 35). So far, no marker of BMSC functionality has been identified making difficult the assessment of the cell-source quality thus complicating successful therapeutic prognosis.

Overall, the donor-to-donor variations combined with the small amount of available BMSC and their limited life-span makes both their potential use difficult and the development of standardized procedures unlikely. Toward this perspective, a suitable cell source for the standard generation of osteogenic graft should maintain the basic properties of BMSC over time, while being of substantial availability.

2.2 Scaffolding material

Scaffolds aim at providing a 3D structure that support cell attachment, proliferation and differentiation. The material offers a template that seeded cells may be able to remodel while depositing an ECM, thus mimicking the reconstruction of their natural environment.

Two types of scaffold can be distinguished whether they are from natural origin (e.g. decellularized bone, collagen-based) or purely synthetic (e.g. titanium, polycaprolactone). It is generally recognized that no ideal scaffold can be defined, even for a specific tissue. As for bone, the diverse roles carried on by a tissue make unlikely the regeneration and subsequent restoration of the ensued functions by the use of a unique material (36). Nevertheless, it is possible to identify and consider key characteristics such as the composition, architecture, structural mechanics, surface properties, or biocompatibility/degradability, in order to envision a suitable material for a specific regeneration purpose (36).

In the context of bone tissue engineering, candidate materials should display key properties such as (i) biocompatibility and biodegradability, (ii) a certain rigidity and porosity (thus resembling the natural bone tissue), (iii) and osteoconductivity, for an optimal integration of the graft (37). Importantly, the presence of a mineral component in the scaffold may be needed, as shown to be notably required for an ectopic *in vivo* bone formation (38).

In this regard, ceramic materials appear extremely promising for bone repair. Ceramic scaffolds can be obtained from decellularized bone (biologic origin), or derived from a manufacturing process (synthetic origin). Synthetic ceramics can be tailored to achieve both a porosity resembling the one of natural bone tissue, as well as an adapted shape perfectly filling the defect site. Ceramics are composed of calcium-phosphate/hydroxyapatite crystals that display a similar size and morphology to that of bone apatite. This results in equivalent biodegradability (39), bioactivity, osteoconductivity (supporting bone ingrowth) and osteoinductivity (inducing bone formation) than bone tissue. For this reasons, ceramics are already used as dental implant (40, 41) and in orthopedic surgery (42, 43). Several studies also report the successful combination of osteoprogenitors and ceramic-based scaffolds for the generation of bone graft (44, 45).

SCAFFOLD	COMPOSITION	PROPERTIES	ORIGIN
Ceramic	CaP, Hydroxyapatite	Bioactive, biocompatible, biodegradabble, osteoconductive, osteoinductive	Biologic, synthetic
Collagen	Collagen type 1	Bioactive, biodegradabble, osteoconductive	Biologic
Metals	Titanium, Nickel, Steel	Bioinert, biocompatible	Synthetic
Polymeric	Polycaprolactone poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers PLGA	Biocompatible	Synthetic
Bioactive glass	CaO-Na_2O-SiO_2, P_2O_5	Bioactive, biodegradable, Biocompatible, osteoconductive	Synthetic
Hydrogels	Fibrin, cellulose, chitosame, polyester, silicon	Bioactive, biocompatible, biodegradable	Biologic, synthetic
Composite materials	Mixed	biocompatible	Synthetic

Table 1. Overview of scaffold used in regenerative medicine (46-49).

2.3 Culture conditions

Following the choice of suitable cell source and scaffolding material, the culture conditions need to be defined as playing an important role in the development of an osteogenic graft.

The medium composition and associated growth factors allow guiding cells toward proliferation and differentiation, as well as promoting the deposition of a rich ECM coating the material. Specific culture conditions have been identified to direct the osteogenic differentiation of cells *in vitro* (50).

Other parameters such as the temperature or the oxygen supply are also of importance as influencing cell differentiation (51).

Culture systems have been also described as playing a central role for the generation of appropriate osteogenic grafts. Importantly, over the last years 3D culture within bioreactor system has emerged as alternative to classic 2D, monolayer culture. The development of such device, and in particular perfusion-based devices, allowed for a better monitoring of the culture conditions (52), while introducing other tuneable variable such as flow rate (mechanical stimulation), which also offers the possibility of a dynamic cell seeding (53, 54). In particular, the strict control of the culture parameters conferred by these devices is in line with the development of clinical-grade conditions, toward the clinical translation of engineered tissue (52).

Initially developed to better mimic the physiologic environment of cells (55), leading to a better preservation of their intrinsic differentiation potential (56), bioreactor systems also allow the formation of more homogeneous tissue (57). The perfusion confers the possibility to provide nutrients supply throughout the graft while removing the toxic cellular waste. Taken together, this allows the development of scaled-up tissue engineered constructs of homogeneous quality, while avoiding the formation of a necrotic core typically appearing in grafts of critical size.

2.4 Vascularization

The metabolic waste removal as well as the supply of oxygen, nutrients and factors is of high importance upon transplantation in order to either allow the survival of the implanted living fraction, or the rapid graft colonization with osteoprogenitors in the case of an acellular graft. Moreover, a proper bone remodelling requires an active process of angiogenesis that supplies the necessary growth factors and stem cells (58). Thus, from the rapid development/recruitment of a vasculature may depend the successful and stable integration of the implanted graft.

Hence, vascularization is a central issue in tissue engineering and its promotion may be needed. Current strategies notably proposed the delivery of angiogenic signals (59) such as Vascular-Endothelial Growth-Factor α (VEGFα), Fibroblast-Growth-Factors (FGFs) or Platelet-Derived-Growth

Factor (PDGF). VEGFα is a potent contributor of angiogenesis, promoting the formation of capillaries, by stimulating the survival, proliferation and migration of endothelial cells (60). FGFs (type 1 and 2) can stimulate the proliferation and differentiation of both endothelial and smooth muscle cells. Instead, PDGF only allow the recruitment of smooth muscle cells to form pre-capillary sphincters, allowing for blood vessel contraction and relaxation (61). Nevertheless, the delivery of growth factors to enhance the vascular network establishment required defined doses and a controlled kinetic of release to achieve a safe therapeutic effect. An improper strategy may indeed result either in an inefficient promotion of vascularization or in the formation of aberrant vessels/angiomas (62). In the context of engineered bone tissue, osteoprogenitors are known to secrete angiogenic factors including VEGF (63), from which a certain amount is entrapped in the deposited ECM (64). Thus, both implanted cells and/or the ECM coating the material are expected to participate in the recruitment of host vessels after implantation.

The co-culture of osteoprogenitors with an endothelial fraction is an alternative strategy. Those vasculogenic cells are expected to form a capillary network (*in vitro* or *in vivo*) (18, 65) that can connect with host vessels, leading to improved survival of the grafted cells. Nevertheless, the use of an endothelial progenitors source suffers from a lack of abundance in biopsy and their isolation is considered clinically unsuitable as complex, multi-tasking and costly (38).

3 Generation of acellular osteogenic grafts

3.1 State-of-the-art living graft

(i) Osteogenicity assessment

Tissue engineering approaches for bone repair consist in the combination of stem cells and an appropriate scaffold, in order to generate an osteogenic material. The best assessment of the osteogenic potential of a human graft is performed through its ectopic (e.g. subcutaneously) or orthotopic (e.g. cranial defect) implantation into immunodeficient animals (66).

Ectopic implantation sites are the most stringent condition as no bone can naturally form in this environment. As a consequence, the eventual induced bone formation can only be attributed to the

cells and/or signals arising from the implant. Orthotopic models are more clinically relevant allowing assessing the intrinsic osteogenicity of an implant. However, in this model the induced bone formation is often the result of both host and grafted cells, making the evaluation of the graft participation in the repair difficult. Importantly, orthotopic site also allows assessing the osteoconductivity of the graft by visualizing the possible construct integration within the host bone.

In both ectopic and orthotopic models, the use of immuno-compromised animals however appears to be a limitation. Beyond the interspecies differences in bone formation, these models might also be problematic in the sense that they do not reflect the inflammatory mechanisms that naturally occur in immunocompetent organism (67).

(ii) State-of-the-art

The generation of *in vitro* tissue was developed in order to obtain graft with enhanced osteogenic properties. The capacity of engineered tissue to promote bone formation was observed in both ectopic (68-70) and orthotopic models (71, 72) using BMSC differentiated on 3D scaffold.

Interestingly, most of the successful study involved the use of ceramic materials, promoting the osteogenic differentiation of seeded cells and also providing the necessary mineral substrate for the formation of bone. In comparison, inert scaffolds without the supply of strong BMPs doses typically fail in generating mature bone tissue at an ectopic implantation site (38).

The relative success of the cell-based tissue engineering has to be tempered by the limited standardization of the graft quality. As the bone formation mainly results from the transplant cells, this strategy is highly dependent of the cell source quality, limiting a full exploitation of this approach. Moreover, despite successful study conducted in large animals, few of them led to clinical translation. One of the key factor involved the survival of the cells following implantation (73). In large bone defect, the rapid vascularization is a requirement to allow implanted cells to form bone. An alternative strategy should not only rely on the cell compartment to induce bone formation and provide the necessary osteogenic and angiogenic factors for a rapid engraftment.

The generation of decellularized grafts represents a promising alternative, as aiming at inducing bone formation through the secreted ECM and embedded factors. Importantly, the removal of the cellular

compartment prior to implantation may also allow the allogeneic exploitation of the tissue, as *off-the-shelf* product. Nevertheless, the success of this strategy entirely relies on the deposition of an osteoinductive ECM by the cultured cells, and its preservation upon decellularization procedure.

3.2 The extracellular matrix

The ECM is a combination of structural and functional proteins, proteoglycans, lipids and crystals that has a unique composition and physical properties for every tissue and organ in the body. Acting as a reservoir for morphogens while providing mechanical support for resident cells, ECM participates in cell communication as well as in defining the shape and stability of tissues (74). ECM cues have been demonstrated to specifically promote cell recruitment, adhesion, migration, proliferation and differentiation in a way that reflects the functional needs and biological identity of tissues (75). In bone, residing cells are known to secrete key factors that serve as inflammatory mediators, matrix-degrading enzymes as well as osteoinductive signals (1). The suitable presentation of those embedded factors may induce instructive processes such as bone-resorption/formation, angiogenesis, or immune cell recruitment.

3.3 Decellularized ECM in regenerative medicine

The concept of using decellularized ECM to induce repair relies on the hypothesis that its instructive elements may retain at least in part their functionality even in the absence of the living cellular component. Based on this rationale, decellularized ECM has received increased attention in the field of regenerative medicine as an *off-the-shelf* and immune-compatible alternative to living grafts for tissue and organ repair. Decellularized ECM is expected to induce regenerative processes through the physiological presentation of different cocktails of regulatory molecules in a mechanically suitable environment.

The instructive ECM-coated scaffold material could be activated by living cells prior to implantation, with the assumption that ECM is capable of directing the differentiation fate of the seeded cells (15, 76, 77). However, in a more attractive paradigm, the decellularized ECM could be directly used to

instruct resident cells towards endogenous tissue repair. The described concept has received convincing proof-of-principle evidences from epithelial (78), musculoskeletal (79), vascular (80), myocardial (81), pulmonary (82), renal (83) and pancreatic tissue engineering (84).

Starting from decellularized native bone as a prototype ECM graft [13], bone tissue engineering has evolved toward the generation of cell-laid coated material. The instructive capacity of those engineered and subsequently decellularized ECM was already demonstrated. ECM deposited *in vitro* by BMSC has been shown to support cell proliferation, prevent spontaneous differentiation and enhance the osteogenic capacity of freshly reseeded BMSCs (85, 86). The osteogenicity of this acellular ECM graft was also demonstrated *in vitro* (87), as efficiently fostering the osteoblastic differentiation of BMSC.

Additional studies report the successful formation of bone by using decellularized ECM combined with hydroxyapatite scaffold, implanted in a rodent critical-sized cranial defect (88, 89). Using bioreactors, the osteogenic performance of a polymeric scaffold was enhanced by the ECM deposited by BMSC. Nevertheless, those constructs were shown not to be capable to induce bone formation when implanted in an ectopic environment. Moreover, the reported bone formation induced by decellularized ECM graft in an orthotopic model was shown to be very limited and the created defect could not be filled despite a substantial *in vivo* period.

Taken together, if the use of decellularized ECM as osteo-inductive material was shown to be a promising strategy, the improvement of the final osteoinductivity appears to be essential. In particular, the use of a cell source with standard properties may be a requirement, as well as the choice of a suitable decellularization technique.

3.4 Existing decellularization strategies

Decellularization protocols aim at removing all cellular material without adversely affecting the composition, mechanical integrity and eventual biological activity of the remaining ECM (90). The effective removal of antigenic epitopes associated with cell membranes and intracellular components of tissues is necessary to minimize or avoid an adverse immunologic response by

allogeneic and xenogeneic recipients of the ECM scaffold material. For this purpose, a variety of methods have been developed, which can mainly be divided into chemical, biological or physical treatments (table 2).

Chemical-based procedures rely on the use of acids and bases, hypertonic/hypotonic solutions or detergents (e.g. Triton-X, Sodium dodecyl sulfate) while biological ones involved the use of enzymes such as trypsin or nucleases. Instead, typical physical treatments consist in temperature variations (freeze & thaw cycles) or mechanical pressure. Protocols described in literature tend to combine several of these principal methods in order to increase the efficiency of decellularization and at the same time reduce damage to the ECM by using less destructive conditions.

All of the above mentioned methods can reach variable degrees of decellularization efficiency, but all fail in preserving the integrity of the ECM, making the development of alternative decellularization strategy a necessity. A standard decellularization method preserving both the structure and composition of an *in vitro* generated graft may support a regenerative response. In fact, the maintenance of the integrity of the ECM following decellularization is expected to support a more predictable, reproducible and effective clinical use of the resulting material through the preservation of the osteoinductive potential of the graft.

Decellularization treatment	Technique	Method	ECM impairment
Chemical treatment	Acids & bases	React with proteins leading to the solubilization of cell components	Protein denaturation, alteration of collagens and glycosaminoglycans (GAG)
	Hypertonic/ hypotonic	Disrupt cells by osmotic shock	Relative efficiency, difficult removal of cellular material
	Detergents	Disrupt DNA-protein, lipid and lipid-protein interactions	Protein denaturation, affect collagens/GAG/growth factors content
Biological treatment	Enzymes	Rupture of peptide bonds anchoring cells to the ECM	Remain entrapped in the ECM, extensive exposition lead to collagen/laminin/ fibronectin/elastin/GAG destruction
Physical treatment	Freeze & Thaw cycles	Cell bursting	Damage the ECM ultrastructure, inactivation of growth factors
	Sonication		

Table 2. Overview of existing decellularization procedures (75, 91-96).

4 Aims of the thesis

4.1 General aims

The use of engineered decellularized tissue appears as a promising strategy to promote bone repair, offering the possibility to induce a repair without immuno-matching requirement. Nevertheless, clear limitations have been identified. First, the development of a cell source displaying stable properties and substantial availability appears to be essential for the generation of standardized ECM-coated material. Moreover, the preservation of the ECM integrity is necessary for eliciting a regenerative response. As existing decellularization protocols vary in their cell removal efficiency and do not allow for a controlled preservation of the structural, biochemical and/or biomechanical features of the engineered tissues, the development of an alternative strategy is a necessity.

My thesis proposes the combined development of (i) a suitable cell source and (ii) a decellularization strategy for the generation of tissue engineered bone graft. The successful association of these key components of the tissue engineering paradigm is expected to lead to (iii) the standardized generation of ECM-coated materials with enhanced properties.

4.2 Chapters content description

The experimental work constitutes three chapters, in the form of scientific publications.

Chapter 2 describes the generation of the first immortalized but death-inducible cell line, aiming at solving cell source availability and standardization issues. For this purpose, a human Mesenchymal Stromal Cell (hMSC) line inducible toward apoptosis was generated, through the implementation of a human telomerase gene and a pre-established inducible suicide device (Bourgine et al., Stem Cell Research, Re-Submitted). The cell line represents a relevant tool for bone tissue engineering applications as displaying a continuous growth while retaining stable differentiation properties. In particular, the integrated suicide system leads to an increase of safety margins, allowing for a temporally controlled survival of cells.

Chapter 3 is a description of a novel decellularization procedure for the generation of acellular tissue with preserved properties. It reports the possibility to decellularize native or engineered tissue by activation of programmed cell death. In particular, the concept of apoptotic-driven decellularization has been explored in the context of bone tissue engineering for the generation of acellular grafts with preserved properties (97).

Finally, chapter 4 reports the successful combination of the generated cell line and the apoptotic-based decellularization procedure for the generation of ECM-coated constructs. The activation of the suicide system integrated in the cell line serves as decellularization method. Using a perfusion-based 3D culture system, the engineered cell line was shown to adhere, proliferate, differentiate and to deposit an ECM when seeded on ceramic scaffold. The resulting generated constructs were directly

and efficiently decellularized by convection of the apoptotic-inducer within the perfusion bioreactor system, leading to the successful generation of decellularized ECM-coated constructs. Importantly, the decellularization by apoptosis was demonstrated to better preserve the matrix integrity as compared to the conventional Freeze & Thaw method (Bourgine et al., manuscript in preparation).

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Chapter 2

“Combination of immortalization and inducible death strategies to generate a human mesenchymal stromal cell line with controlled survival”

Enclosed is the manuscript submitted to Stem Cell Research

Combination of immortalization and inducible death strategies to generate a human mesenchymal stromal cell line with controlled survival

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Key words. Mesenchymal stromal cells ● Immortalization ● apoptosis ● Inducible death

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Abstract

The hTERT-immortalization of human bone marrow-derived Mesenchymal Stromal Cells (hMSCs) was proposed to address availability/standardization issues for experimental or clinical studies, but raised concerns due to possible uncontrolled growth or malignant cell transformation.

Here we report a method to generate a hMSCs line with controlled survival, through the implementation of a pre-established suicide system (inducible caspase 9, iCasp9) in hTERT-transduced hMSCs.

Primary hMSCs were successfully immortalized (>280PD) and further transduced with the iCasp9 device. A clone was selected and shown to maintain typical properties of primary hMSCs, including phenotype, differentiation and immunomodulation capacities. The successive transductions did not induce tumorigenic transformation, as assessed by analysis of cell cycle regulators and *in vivo* luciferase-based cell tracking. Cells could be efficiently induced toward apoptosis (>95%), even upon differentiation.

By combining the opposite concepts of `induced-life' and `inducible-death', we generated a hMSCs line with defined properties and allowing for temporally controlled survival. The cell line represents a relevant tool for medical discovery in regenerative medicine and a potential means to address availability, standardization and safety requirements in cell & gene therapy. The concept of a hTERT-iCasp9 combination, here explored in the context of hMSCs, could be extended to other types of progenitor/stem cells.

Introduction

Human bone marrow-derived Mesenchymal Stem/Stromal Cells (hMSCs) are widely investigated in regenerative medicine due to their multilineage differentiation capacity (Pittenger, Mackay, Beck, Jaiswal, Douglas, Mosca, Moorman, Simonetti, Craig, and Marshak, 1999), immunomodulatory properties (Nauta and Fibbe, 2007), as well as their ability to migrate and deliver regulatory factors at injured sites or within tumour stroma (Kidd, Spaeth, Dembinski, Dietrich, Watson, Klopp, Battula, Weil, Andreeff, and Marini, 2009). Despite the large scientific interest and broad range of potential therapeutic applications, the coherent pre-clinical and clinical use of hMSCs is challenged by several critical issues, including the limited availability and the variability among different donors (Phinney, Kopen, Righter, Webster, Tremain, and Prockop, 1999).

The low amount of hMSCs in bone marrow aspirates (0.001 to 0.1% of nucleated cells) typically necessitates an *in vitro* expansion phase prior to use. The phase of amplification is limited by the replicative senescence phenomenon occurring under *in vitro* culture conditions, typically after 30 to 40 population doublings (PD) (Stenderup, Justesen, Clausen, and Kassem, 2003). Along with serial passages, the differentiation potential of hMSCs is reduced and functional changes have been reported (Minguell, Erices, and Conget, 2001;Siddappa, Licht, van, and de, 2007a). The need for extensive hMSCs expansion, combined with their short life-span, is aggravated by the high variability among preparations from different donors, even if in the same age range (Siddappa, Licht, van, and de, 2007b).

To overcome hMSCs standardization and supply based problems, the generation of immortalized hMSCs was developed through the insertion of a human telomerase catalytic subunit (hTERT) (Boker, Yin, Drosse, Haasters, Rossmann, Wierer, Popov, Locher, Mutschler, Docheva, and Schieker, 2008;Huang, Zheng, Sun, Guo, Yang, Chen, Xu, Wang, Shen, Pan, Jin, and Wang, 2008;Jun, Lee, Cho, Suh, and Jung, 2004;Piper, Wang, Yamamoto, Malek, Luu, Kuo, and Kim, 2012;Simonsen, Rosada, Serakinci, Justesen, Stenderup, Rattan, Jensen, and Kassem, 2002a). The generation of hTERT-MSCs lines allowed extended hMSCs lifespan (>300PD) and was shown to preserve some of the properties of primary hMSCs, such as their multilineage differentiation potential (towards osteogenesis, chondrogenesis and adipogenesis) (Abdallah, Haack-Sorensen, Burns, Elsnab, Jakob,

Hokland, and Kassem, 2005;Jun *et al.*, 2004;Mihara, Imai, Coustan-Smith, Dome, Dominici, Vanin, and Campana, 2003) and bone formation capacity (Simonsen, Rosada, Serakinci, Justesen, Stenderup, Rattan, Jensen, and Kassem, 2002b).

The enthusiasm raised by the availability of an unlimited cell source with stabilized properties was however tempered by safety aspects. A strong telomerase expression was in fact associated with many cancer types (Counter, Hahn, Wei, Caddle, Beijersbergen, Lansdorp, Sedivy, and Weinberg, 1998;Latil, Vidaud, Valeri, Fournier, Vidaud, Lidereau, Cussenot, and Biache, 2000), and neoplastic changes have been reported following hTERT-transformation of hMSCs (Burns, Abdallah, Guldberg, Rygaard, Schroder, and Kassem, 2005;Serakinci, Guldberg, Burns, Abdallah, Schrodder, Jensen, and Kassem, 2004) resulting in uncontrolled cell growth. Such risk may be aggravated if hMSCs are delivered within a 3D matrix as opposed to systemic infusion, due to the previously reported enhanced long-term engraftment (Daga, Muraglia, Quarto, Cancedda, and Corte, 2002).

An elegant approach was recently proposed to enhance the safety of cellular therapies by the use of an inducible death-system (iDS) (Ramos, Asgari, Liu, Yvon, Heslop, Rooney, Brenner, and Dotti, 2010;Straathof, Pule, Yotnda, Dotti, Vanin, Brenner, Heslop, Spencer, and Rooney, 2005), based on the expression of a modified caspase 9 (iCasp9).

Beyond safety considerations, the introduction of an iDS may additionally offer the unprecedented opportunity to temporally control the survival of the transduced cells, in order to investigate their functional role in *in vitro* or *in vivo* models. However, while the device demonstrated a great efficiency in primary cells (Ramos *et al.*, 2010;Straathof *et al.*, 2005), no study has yet reported its functionality in immortalized cells. The concept may in fact be challenged by the fact that a hTERT over-expression has been previously associated to cell-suicide inhibition (Deeb, Gao, Liu, Kim, Pindolia, Arbab, and Gautam, 2012;Indran, Hande, and Pervaiz, 2011;Liang, Ye, Dai, Shen, and Xu, 2012), conferring notably a resistance to caspase-mediated apoptosis (Bermudez, Erasso, Johnson, Alfonso, Lowell, and Kruk, 2006).

Towards the standardized, controlled and versatile use of hMSCs, we then aimed at assessing the feasibility to associate immortalization and `inducible-death' concepts to generate a hTERT-hMSCs line efficiently inducible to apoptosis and maintaining the typical properties of primary hMSCs. The

successful hTERT and iDS combination is expected to lead to the generation of an unlimited and well characterized hMSCs source with controlled survival, opening a variety of applications for research and pre-clinical purposes.

Materials and Methods

Cell culture

Cell isolation

Human bone marrow aspirates were obtained during routine orthopedic surgical procedures involving exposure of the iliac crest, after ethical approval (EKBB, Ref.78/07) and informed donor consent. Marrow aspirates (20 ml volumes) were harvested from a healthy donor (female, 51 year's old) using a bone marrow biopsy needle inserted through the cortical bone and immediately transferred into plastic tubes containing 15,000 IU heparin. After diluting the marrow aspirates with phosphate buffered saline (PBS) at a ratio of 1:4, nucleated cells were isolated using a density gradient solution (Histopaque, Sigma Chemical, Buchs, CH). Complete medium consisted of either Dulbecco's modified Eagle medium (DMEM) or α-minimum essential Medium (αMEM) with 10% fetal bovine serum, 1% HEPES (1M), 1% Sodium pyruvate (100mM) and 1% of Penicillin-Streptomycin-Glutamin (100X) solution (all from Gibco). Nucleated cells were plated at a density of 3.10^6cells/cm2 in complete medium supplemented with 5 ng/ml of fibroblast growth factor-2 (FGF-2, R&D Systems) and cultured in a humidified 37C°/5% CO_2 incubator. In immortalized cell cultures, FGF-2 supply was interrupted after 140 PD, due to both the steady proliferation observed even in its absence and the possible impairment of the multi-lineage potential of hMSCs (Lai, Krishnappa, and Phinney, 2011). Medium was changed twice in a week. HMSCs were selected on the basis of adhesion and proliferation on the plastic substrate 1 week after seeding.

Cell engineering

Immortalization

Immortalization was performed by the use of a lentivirus Lenti-hTERT-eGFP (LG508, Biogenova). Infection of hMSCs was performed 1 week after isolation and plating of the nucleated cell fraction from bone marrow aspirate. The virus was delivered at a Multiplicity Of Infection (MOI) of 5 in complete medium supplemented with 8µg/mL of polybrene (Sigma Aldrich). The MOI was selected following preliminary experiments, in order to obtain a high transduction efficiency while preserving the cell functionality. The success of immortalization was assessed by flow cytometry (eGFP expression), telomerase activity measurement (TraP assay, Millipore, cat# S7700), a senescence assay (β-galactosidase assay, Sigma Aldrich, cat# CS0030) and by following the population doublings (PD) of the cells. The formula ($PD_{(n/(n-1)}$=(log(N_n/N_{n-1}))/log2 was used for the calculations of the PD at passage n, based on the number N of counted cells. The cumulative population doubling levels (PDL) is the sum of population doublings (PD) across each serial passage.

Retrovirus production

The retro-vector carrying the modified caspase 9 and CD19 (iCasp9-ΔCD19) was kindly provided by Dr. Carlos Almeida Ramos (Baylor College of Medicine, Houston, Texas, USA). The retrovirus was produced after transfecting the phoenix ECO cell line (American Type Culture Collection, cat# SD3444;) with the iCasp9-ΔCD19 vector. Virus containing supernatant was collected every 12 hours, passed through a 0.45 µm filter and conserved at -80°C.

The MSCV Luciferase PGK-hygro plasmid (Addgene plasmid 18782) was used for the production of retrovirus carrying the luciferase system (retro-Lucif). The virus production was performed using the same protocol as for the iCasp9-ΔCD19 retrovirus. Cells were selected by hygromycin B (Sigma, cat# H3274) treatment for 2 weeks.

Retroviral transduction

HMSCs were plated at 6000 cells/cm^2 in 60-mm dishes the day preceding the transduction. Cells were transduced by incubation with retroviral vector supernatants supplemented with 8 µg/ µL of polybrene

(SigmaAldrich) for 5 minutes at 37°C and centrifuged at 1100g for 30 min at room temperature in the dishes, followed by fresh medium replacement.

Cell sorting

HMSCs stably expressing hTERT-eGFP and/or iCasp9-ΔCD19 were purified using a FACS-Vantage SE cell sorter (Becton Dickinson, Basel, Switzerland). Cells were sorted after immortalization and iCasp9-ΔCD19 transduction at 30 PDL and 195 PDL respectively. Prior to sorting of the CD19 positive fraction, cells were labeled using a human anti CD19-PerCP antibody (BD biosciences, cat# 561295).

Cell Characterization

Cell phenotyping

HMSCs phenotype was determined by cytofluometry analysis with fluorochrome-conjugated antibodies to human CD44 (cat# 559942), CD29 (cat# 555443), CD73 (cat# 560847), CD90 (cat# 559869), CD34 (cat# 555822), CD45 (cat# 555483), CD146 (cat# 550315), CD19 (cat# 561295), Epcam (cat# 347200) all from BD Pharmingen, and E-cadherin (cat#FAB18381P) from R&D Systems.

Adipogenic differentiation

HMSCs were differentiated as previously described (Barbero, Ploegert, Heberer, and Martin, 2003;Jaiswal, Haynesworth, Caplan, and Bruder, 1997;Jakob, Demarteau, Schafer, Hintermann, Dick, Heberer, and Martin, 2001). Briefly, cells were seeded at 3000 cells/cm^2 and cultured for 1 week in DMEM complete medium without passage. During the following 2 weeks, cells were exposed to four differentiation cycles consisting in alternating `strong' adipogenic medium (Dexamethasone $10^{-6}$ M, Indomethacin, Insulin, IBMX) for 3 days and `light' adipogenic medium (Insulin) for 1 day.

Osteogenic differentiation

HMSCs were seeded at 3000 cells/cm^2 and differentiated for 3 weeks in osteogenic medium (OM). Osteogenic medium consists in αMEM complete medium supplemented with 10 nM Dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate and 10 mM β-glycerophosphate (Maniatopoulos, Sodek, and Melcher, 1988).

Chondrogenic differentiation

Culture of hMSCs was performed in 0.5 ml of DMEM containing 4.5 mg/ml D-Glucose, 0.1mM nonessential amino acids, 1mM sodium pyruvate, 100mM HEPES buffer, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.29 mg/ml L-glutamine supplemented with 0.1mM ascorbic acid 2-phosphate, 10 ng/ml TGFb1 and 10^{-7} M dexamethasone (Chondrogenic medium) as previously described (Jakob *et al.*, 2001). Cells (350,000) were centrifuged in 1.5 ml conical polypropylene tubes (Sarstedt, Numbrecht, Germany) to form spherical pellets. After 2 weeks of culture, pellets were processed for histology and gene expression analysis.

Real-time PCR

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA), treated with DNAse and retrotranscribed into cDNA, as previously described (Frank, Heim, Jakob, Barbero, Schafer, Bendik, Dick, Heberer, and Martin, 2002). Real-time PCR was performed with the ABIPrism 77000 Sequence Detection System (Perkin Elmer/Applied Biosystem, Rotkreuz, Switzerland) and expression levels of genes of interest were normalized to GAPDH. Primers and probe sets of chondrogenic and osteogenic genes were used as previously described (Frank *et al.*, 2002). Human PPARγ (Hs00234592_m1) Retinoblastoma 1, p53 (Hs01034249), p21 (Hs00355782_m1) primers and probe were provided by Applied Biosystem. Probe and primers for c-Myc were ordered from Mycrosynth© and designed as follow:

forward GCCACGTCTCCACACATCAG

reverse TCTTGGCAGCAGGATAGTCCTT

probe ACGCAGCGCCTCCCTCCACTC

Biochemical Stainings

Alizarin red, oil red-O and Alcian blue biochemical stainings were used to respectively assess the osteogenic, adipogenic and chondrogenic differentiation of the cells, as previously described (Barbero *et al.*, 2003;Jaiswal *et al.*, 1997;Jakob *et al.*, 2001).

Tumorigenicity assay

One million M-SOD cells previously transduced with the retro-Lucif virus were mixed with Matrigel Matrix (BD Biosciences) in a ratio 1:1 (V/V) with PBS and injected subcutaneously into the left flank of NOD/SCID mice (8-10 weeks old mice), initially obtained by Charles River Laboratories (Germany). Eventual tumor growth was monitored both weekly by palpation and by *in vivo* luminescence signal acquisition using an ISIS luminometer system. Prior to acquisition, mice received an intraperitoneal injection of 300µL of D-Luciferin Potassium salt (Goldbiotechnology) at 15 mg/mL. Cells were tracked for a period of 6 months post-injection.

In vivo bone formation

In vivo ectopic bone formation was assessed as previously described (Braccini, Wendt, Jaquiery, Jakob, Heberer, Kenins, Wodnar-Filipowicz, Quarto, and Martin, 2005). Briefly, cells were resuspended in 30µl of Fibrinogen (20 mg/ml Baxter, Austria), mixed with 30µl of Thrombin (6 IU/ml Baxter, Austria) and immediately loaded onto 35µg of bovine bone-derived granules (Actifuse, Apatech, Baxter, Austria). The constructs were transferred for 15 minutes in a humidified incubator at 37 °C with 5% CO2 to allow fibrin polymerization and implanted subcutaneously in CD-1 nu/nu nude mice (Charles River, Germany). After 8 weeks, the constructs were harvested, fixed overnight in 4% formalin, decalcified with a decalcification solution made of 7% EDTA (Sigma, cat# E5134) and 10% sucrose (Sigma, cat# S9378), paraffin embedded and 5µm-thick-sections were obtained from different levels. Sections were stained with Masson's trichrome and observed microscopically to detect histological features of bone tissue. For fluorescence analysis, samples were fixed in 1.5% paraformaldehyde, decalcified with 7% EDTA (Sigma, cat# E5134), embedded in optimal cutting

temperature, and snap frozen in liquid nitrogen. Sections (5 μm thick) were incubated with DAPI to stain nuclei. Fluorescence images were acquired using an Olympus BX-61 confocal microscope.

Immunomodulation assay (CFSE)

The immunomodulation of hMSCs was assessed as previously described (Gattinoni, Lugli, Ji, Pos, Paulos, Quigley, Almeida, Gostick, Yu, Carpenito, Wang, Douek, Price, June, Marincola, Roederer, and Restifo, 2011). Briefly, hMSCs were seeded in 96-well plates at 20.000, 10.000, 5000 and 2000 cells/well. CD4+ lymphocytes were extracted from whole blood PBMCs by magnetic beads labeling (Miltenyi Biotec) and seeded on top of hMSCs at 1*10^5 cells per well after labeling with 2 μM CFSE (7 min at 37 °C). The CD4+cells were left untreated or stimulated with 1 μg/mL of phytohemagglutinin and the proliferation index was determined after 4 days by flow cytometry and analyzed with FlowJo software (Treestar).

Death induction

The B/B homodimerizer (Clontech, cat# 635060) was added at 50 nM to iCasp9-ΔCD19 transduced HMSCs in culture medium to activate the apoptosis pathway through the dimerization of the modified caspase 9. Percentage of induced death was assessed 12 hours later by FACS analysis, after cell harvest and staining with Annexin V-APC (BD Biosciences, cat#550475) and Propidium Iodide (PI, BD Biosciences, cat# 51-66211E) in Annexin-V binding buffer (BD Biosciences, cat# 556454). Control cells were cultured in the same medium without exposure to the homodimerizer.

Statistical analysis

Data are presented as means ± standard error. The significance of differences was evaluated using analysis of variance (ANOVA) followed by the Bonferroni test for every set of data on which multiple comparisons were performed. For single comparison, Mann-Whitney test was used. $P < 0.05$ was considered to indicate statistical significance.

Results

Immortalization of human bone marrow derived Mesenchymal stromal cells

Primary hMSCs were transduced with a lentivirus carrying the human telomerase gene (figure 1A). The rate of transduction was assessed by flow cytometry through the enhanced GFP (eGFP) reporter gene. After transduction, 78% of the cell population expressed the transgene. The eGFP positive fraction was sorted to increase the purity of the transduced population (Supp. data 1). The immortalized population (hTERT-hMSCs) stably displayed higher telomerase activity (4.4 ±1.5 fold) than their primary counterpart (primary hMSCs), even after extensive proliferation (shown up to 150 doublings, figure 1B). Primary hMSCs did not proliferate for more than 35 PD, while the immortalized hMSCs underwent more than 270 PD (figure 1C). Primary cells became senescent after 35 PD as assessed by β-galactosidase staining, while the transduced population bypassed the senescence associated crisis (figure 1D). The clonogenicity of the immortalized population was about 8-fold higher than for primary hMSCs (9.3% vs 80.3%). A stable hTERT-hMSCs line with steady proliferation capacity was thus successfully generated.

Generation of a clonal hTERT-iDS hMSCs line

After 170 PD, the hTERT-hMSCs line was transduced with the retrovirus carrying an inducible death system (iDS), including CD19 as reporter gene (figure 1E). After one round of transduction, more than 50% of the cells expressed CD19. After sorting, more than 98% of the cells expressed the reporter gene (figure 1F), resulting in the isolation of a stable hMSCs line uniformly carrying the death-inducible system (hTERT-iDS). The functionality of the iDS was investigated by exposing the hTERT-iDS line to the Chemical Inducer of Dimerization (CID), leading to the activation of the suicide system. Following overnight exposure to CID (figure 1G and supplemental online video 1), more than 99% of the cells were pushed toward death (PI-positive or Annexin-V-positive). No toxicity was induced by exposing primary hMSCs or hTERT-hMSCs to CID. An immortalized and death-inducible hMSCs line was thus successfully generated, demonstrating the possibility to combine a hTERT overexpression with an efficient iDS-mediated killing.

The immortalization process, combined with the insertion of the iDS and the extensive number of PD, gave rise to a likely heterogeneous cell line. In order to obtain a more uniform population, thirty single colonies were picked and 24 of those could be successfully expanded. We then arbitrarily decided to select the clone with the most prominent osteogenic differentiation capacity, as this is considered the common, default differentiation pathway for hMSCs (Muraglia, Cancedda, and Quarto, 2000). Following culture for 3 weeks in osteogenic differentiation medium, the clone displaying the strongest intensity of alizarin red staining was selected (Supp. Data 2). The immortalized hMSC clone carrying the iDS was then denominated as the Mesenchymal-Sword Of Damocles (M-SOD) cell line for further characterization.

Phenotypic analysis of primary hMSCs and the generated cell line(s)

The phenotype of the cell line was cytofluorimetrically assessed at different doublings and after each genetic modification (table 1). All transformed cells expressed typical hMSCs markers (positivity for CD44, CD29, CD73, and CD90) while being negative for both the hematopoietic markers CD34 and CD45 and the epithelial markers EpCam and E-cadherin. The immortalized population was also shown to increasingly express the CD146 marker following FGF-2 removal from the supplemented medium (at 140 PD), consistently with a previous study (Sacchetti, Funari, Michienzi, Di, Piersanti, Saggio, Tagliafico, Ferrari, Robey, Riminucci, and Bianco, 2007). The successive genetic modifications and extensive proliferation of the cell line was thus shown to not lead to an alteration of the mesenchymal phenotype, as assessed by conventional markers.

Differentiation capacity of M-SOD line

The multilineage differentiation capacity of the M-SOD line was tested after different numbers of population doublings (ranging from 250PD to over 280PD) in order to investigate the stability of M-SOD properties. Data presented below are the average of 4 different experimental runs.

After 3 weeks of culture in osteogenic medium (OM), M-SOD cells deposited a thick mineralized matrix, as demonstrated by positive alizarin red staining (figure 2A). A strong induction of key

osteogenic genes (figure 2B) was detected after 2 weeks of culture in osteogenic conditions (94.8-, 8.5-, 130.9-, 16.1- and 58.7-fold for ALP, BMP-2, BSP, OC and RunX2 genes respectively, $p<0.05$). The M-SOD line was thereby shown to be capable to efficiently differentiate toward the osteogenic lineage.

After 3 weeks of culture in adipogenic medium, M-SOD cells were successfully differentiated into adipocytes, as revealed by the positive Oil red-O staining of the lipid droplets (figure 3A). The upregulation of the adipogenic PPARγ gene (16.2-fold, figure 3B, $p<0.05$) confirmed the capacity of the M-SOD line to differentiate toward the adipogenic lineage.

The chondrogenic differentiation capacity of the M-SOD line was assessed by Alcian blue staining after pellet culture in complete or chondrogenic medium (figure 3D). M-SOD cells did not generate a GAG-rich cartilaginous matrix and did not significantly upregulate Sox-9 (data not shown) or type II Collagen, despite a marked increase in type X Collagen gene expression level (291-fold, figure 3E, $p<0.05$). Nevertheless, the primary donor from whom the M-SOD line was derived was also not capable to differentiate toward the chondrogenic lineage (Supp. data 3). This suggests that M-SOD low chondrogenic capacity may not be due to the successive genetic modifications or the extensive doublings.

Death induction upon osteogenic and adipogenic differentiation

The chromatin remodeling that occurs during cell differentiation (Benayahu, Akavia, and Shur, 2007;Siddiqi, Mills, and Matushansky, 2010) can potentially lead to the silencing of the implemented iDS. Consequently, the functionality of the iDS system had to be demonstrated also during cell differentiation.

The death induction efficiency of the M-SOD line was tested during osteogenic or adipogenic differentiation, using cells expanded for different numbers of population doublings (ranging from 250 to over 280). Data presented below are the average of 3 different experimental runs.

During osteogenic differentiation, cells were induced with CID after 1, 2 or 3 weeks of culture in osteogenic medium. After an overnight exposure to CID, a very high killing efficiency was reached

(97.7 ±2.1%, figure 2C, $p<0.0001$) irrespective of the cell differentiation stage. Similar results were obtained by inducing M-SOD cells during adipogenic differentiation (97.1 ±2.5%, figure 3C, $p<0.0001$). The iDS implemented within the M-SOD cell line thus remained highly efficient independently of the differentiation status.

Bone formation capacity of the M-SOD line

The bone formation capacity was assessed by combining 1 million of M-SOD cells together with ceramic granules, in a fibrinogen/thrombin gel, followed by ectopic implantation in nude mice (n=3) for 8 weeks. Samples were retrieved, fixed and decalcified prior to sectioning and histological analysis. The cells secreted a dense collagen matrix and the formation of bone nodules was observed within the constructs, as demonstrated by Masson's trichrome staining (figure 4A). GFP positive cells were observed at the outer regions of those nodules, indicating their contribution in the deposition of the osteoid tissue (figure 4B).

Immunomodulation capacity

HMSCs are known to exhibit immunomodulatory properties, which include the regulation of T-cells proliferation. This intrinsic hMSCs capacity has been proposed to be critical towards hMSCs application for graft versus-host or autoimmune diseases.

To assess whether the M-SOD line could regulate activated lymphocyte proliferation, CD4+ cells were labeled with CFSE and co-cultured with M-SOD cells at different ratios. The proliferation of CD4+ cells was measured 4 days post activation with phytohemagglutinin (PHA), by flow cytometry (figure 4C). In the absence of PHA, CD4+ cells did not proliferate, independently of the presence or not of M-SOD cells, implying that M-SOD cells alone did not trigger an immune reaction. In the presence of PHA but in absence of M-SOD, CD4+ got activated and strongly proliferated (>80%), as shown by the 5 peaks of fluorescence observed, corresponding to 4 division cycles (figure 4D). M-SOD cells were able to inhibit such proliferation in a dose dependent manner (figure 4C). In particular, a 1:5 CD4+/M-SOD ratio resulted in only one division of CD4+ cells (figure 4D). The M-SOD cell line thus displayed antiproliferative properties on activated CD4+ lymphocytes. In this regard, although further

in vivo studies would be required to fully assess the regulatory properties, to the best of our knowledge the M-SOD line is the first reported bone-marrow derived hMSCs line capable of immunomodulation.

Oncogenes expression level and tumorigenicity assessment

Cell immortalization, as any genetic modification, can result in a malignant transformation by impairing the cell cycle regulation. Key proteins, such as p53, p21, retinoblastoma-1 or c-Myc, are known to play an important role as cell cycle regulators, keeping cell proliferation and DNA replication under control (Sharpless and Depinho, 2004). The genes p53, p21, retinoblastoma-1 (Rb-1) and c-Myc were expressed to a similar extent in M-SOD and the primary hMSCs (figure 5A), as shown by the limited fold differences (2.0 ±0.4, 3.3 ±3.1, 1.3 ±0.2 and 2.5 ±0.6 fold for p53, p21, Rb-1 and c-Myc respectively). The M-SOD cell line thus keeps expressing normal levels of cell cycle regulator genes.

The tumorigenicity of the M-SOD cell line was then investigated *in vivo* by subcutaneous injection in the flank of NOD/SCID mice of one million M-SOD cells, previously transduced with a luciferase reporter system (retro-Lucif). Within few days, the luminescence intensity remained focalized and rapidly decreased, suggesting the non-proliferation or dissemination of M-SOD cells (figure 5B). After more than 180 days post-injection, neither luminescence nor tumor formation was detected, indicating the non-tumorigenicity of M-SOD cells *in vivo*.

Discussion

By combining two typically opposing concepts, namely immortalization and death-induction, we generated a hMSCs line with standardized properties and temporal control over survival. In particular, the M-SOD line was shown to: a) steadily proliferate, b) differentiate toward the osteogenic and adipogenic lineages, c) maintain the *in vivo* bone forming capacity, d) regulate the proliferation of activated lymphocytes and e) be efficiently inducible toward death using a clinically approved compound (Ramos *et al.*, 2010).

This study demonstrates the feasibility to induce an optimal killing of hMSCs despite their previous immortalization. The finding is not obvious, since the hTERT expression was previously shown to reduce the activation of caspases 3, 8, and 9, and of pro-apoptotic mitochondrial proteins, while increasing the expression of the anti-apoptotic mitochondrial protein Bcl-2 (Deeb *et al.*, 2012;Indran *et al.*, 2011;Liang *et al.*, 2012). The constitutive expression of the inducible caspase 9 combined with its downstream role in the apoptotic pathway, allowed to bypass the hTERT-associated resistance to apoptosis (Deeb *et al.*, 2012;Indran *et al.*, 2011;Liang *et al.*, 2012), ensuring a control over the cell survival in both un-differentiated and differentiated states.

The M-SOD properties appeared to derive from the primary cells. In fact, the strong osteogenic potency and limited chondrogenic potential could be related to the selection of the M-SOD clone as the one with the highest osteogenic differentiation capacity and to the poor chondrogenic ability of the primary hMSCs. Further studies are needed to confirm that the use of a primary donor or of a clone with a higher chondrogenic capacity would lead to the generation of a line capable to more efficiently undergo chondrogenesis.

The use of immortalized hMSCs with stabilized properties offers standardization opportunity for research purposes. This is in contrast to primary hMSCs, which after extensive *in vitro* culture undergo senescence or possibly degenerate into malignant cells (Ben-David, Mayshar, and Benvenisty, 2011;Stenderup *et al.*, 2003;Tarte, Gaillard, Lataillade, Fouillard, Becker, Mossafa, Tchirkov, Rouard, Henry, Splingard, Dulong, Monnier, Gourmelon, Gorin, and Sensebe, 2010). The disposal of an unlimited cell source also allows a clonal screening and extensive characterization, as well as the long-term monitoring of the population. This might be particularly of interest when introducing further genetic modifications, allowing a full characterization from the integration site to the stability and expression level of the transgene.

Despite the random virus-derived integrations, known to potentially lead to malignant transformations (Du, Jenkins, and Copeland, 2005;Nienhuis, Dunbar, and Sorrentino, 2006), the M-SOD was demonstrated to be non-tumorigenic, both by assessment of the expression levels of cell proliferation and DNA replication gatekeepers, as well as by a standard *in vivo* test. The method can also be further

optimized by combining hTERT and the iDS in a same lentivector, under the control of a unique promoter. Eventually, placing the hTERT gene behind the iDS at the 3' end can ensure the systematic hTERT silencing in case of chromatin remodelling (through either a silencing in 5' of the promoter or the direct silencing of hTERT in 3'). This principle would reduce the number of transductions and guarantee an intrinsic control of successfully immortalized cells by the iDS. Taken together, the non-tumorigenic engineering of M-SOD cells, combined with the control ensured by the iDS, support the relevance of the developed strategy as a first step in the perspective of a clinical use. In this regard, safety concerns would anyway require to be more comprehensively addressed by a panel of additional methods (e.g. karyotypic or micro-array analysis), in order to propose the iDS-hTERT strategy for clinical purposes.

The diversity of hMSCs applications in regenerative medicine makes the M-SOD line an attractive experimental tool for a variety of contexts (figure 6). Beyond representing a standard and stable hMSCs source, the iDS activation could serve for the selective and temporally controlled cell elimination. This would be relevant in *in vitro* models, e.g. to study stage-specific cell-cell conditioning in co-culture systems, or in *in vivo* models, e.g. to address the question of how long the living hMSCs fraction is required in order to induce specific regenerative/tumor targeting effects or to be effective in the treatment of immune-related disorders. The M-SOD line can also be seen as a cellular platform that can undergo additional genetic modifications to overexpress defined factors and thus acting as drug carrier. This could be especially relevant to study the treatment of cancer, inflammatory or autoimmune diseases through the secretion of e.g. TRAIL or IFN-γ (Dai, Moniri, Zeng, Zhou, Rayat, and Warnock, 2011;Yang, Wu, Mao, Bao, Gao, Zhou, Xie, Zhou, and Zhu, 2009). In this regard, the induction of the iDS offers the possibility to switch off the expression of implemented transgenes and thus to temporally control the factor delivery. The efficiency of killing of the M-SOD in vivo could be inferred based on a previous study using the same iDS and analysis of residual cells by luciferase detection (). However, a stringent and quantitative assessment of the *in vivo* killing efficiency would require further transformation of the M-SOD line to express a gene allowing for their sensitive, non-invasive *in vivo* tracking (e.g., production of species-mismatched erythropoietin (Daga *et al.*, 2002)), which goes beyond the scope of the present work.

Finally, the M-SOD line represents a unique tool to engineer devitalized cell-laid matrices with the capacity to induce or regulate regenerative processes, e.g. to promote bone formation (Sadr, Pippenger, Scherberich, Wendt, Mantero, Martin, and Papadimitropoulos, 2012). The M-SOD line would address standardization issues but also allow the devitalization of the engineered matrices upon iDS activation, prior to off-the-shelf storage. As compared to existing devitalization methods (e.g., Freeze & thaw cycles or the use of detergents), the iDS would better preserve the matrix integrity while leading to an efficient killing of the cells in a streamlined manufacturing process.

Conclusions

The present study proposes and validates the concept of the first death-inducible cell line. Although the findings have been generated in the specific context of hMSCs, the paradigm can be extended to other cell types for distinct scientific and/or therapeutic applications. The work represents the basis towards the general perspective of using genetic tools not only to modify or understand cellular function, but also to standardize and increase the safety of cell-based therapies.

Acknowledgements

We are grateful to Dr. Carlos A. Ramos (Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, USA) for kindly providing us with the iDS retrovector. We also thank Francesca Amicarella and Emanuele Trella (Oncology, Department of Biomedicine, Basel University Hospital, Basel, Switzerland) for their help regarding the immuno-modulation experiments. This work was supported by the European Community's Seventh Framework Program (MultiTERM, grant agreement number 238551). Financial support for JG was kindly provided by Dr. Andrea Banfi, Cell & Gene Therapy, Department of Biomedicine, University Hospital Basel, within the EU-FP7 project 'ANGIOSCAFF' (CP-IP 214402).

Disclosure of potential conflicts of interests

The authors declare no potential conflicts of interest.

List of abbreviations

ALP: Alkaline Phosphatase
AM: Adipogenic Medium
BMP-2: Bone Morphogenetic Protein 2
BSP: Bone SialoProtein
ChM: Chondrogenic Medium
CID: Chemical Inducer of Dimerization
CM: Complete Medium
CMV: CytoMegaloVirus
Coll II: Collagen type 2
Coll X: Collagen type 10
DAPI: 4',6-diamidino-2-phenylindole
Epcam: EPithelial Cell Adhesion Molecule
E-cadherin: Epithelial cadherin
FACS: Fluorescence-Activated Cell Sorting
FITC: Fluorescein IsoThioCyanate
GAG: GlycosAmynoGlycan
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
hMSCs: Human Mesenchymal Stromal Cells
hTERT: human TElomerase Reverse Transcriptase
iDS: inducible Death System
Igg: Immunoglobulin G
LTR: Long Terminal Repeat
MOI: Multiplicity Of Infection
M-SOD: Mesenchymal Sword Of Damocles
OC: Osteocalcin
OM: Osteogenic Medium
PD: Population Doubling
PerCP: PERidinin Chlorophill Protein
PPARγ: Peroxysome proliferator activated receptor gamma
RunX2: Run related transcription factor 2

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Figure legends

Table 1: Cytofluorimetric analysis of the primary cells and the generated cell line at different development time. The generated cell lines stably expressed conventional markers for hMSCs (CD44, CD29, CD73, CD90) while being negative for hematopoietic (CD34, CD45) and epithelial markers (Epcam, E-cadherin). The eGFP and CD19 expression respectively reflect the successful immortalization and iDS insertion. The successive genetic transformations and extensive doublings did not impair the mesenchymal phenotype of the cells.

Figure 1: Generation of hTERT-iDS hMSCs line

A) Functional map of the lenti-hTERT-eGFP (Biogenova, LG508) vector use for the immortalization of primary hMSCs. The system consists in a human telomerase gene and a eGFP reporter gene, under the control of separated CMV promoters.

B) Telomerase activity of primary hMSCs and the immortalized population (Trap assay). The immortalized cells (hTERT-MSCs) displayed a higher telomerase activity (> 4-fold) than their primary counterpart. Despite extensive doublings, the telomerase activity of the immortalized population remained stable (respectively assessed at 50, 100 and 150PD).

C) Population doubling levels of primary (black stars) and immortalized hMSCs (white dots). After 35 doublings, the primary cells stopped to proliferate while the M-SOD cells by passed the senescence-associated crisis. After 170PD, the inducible death system (iDS) was introduced in the immortalized population. The M-SOD population underwent more than 270 PD after more than one year of culture.

D) β-galactosidase assay of primary hMSCs (35PD) and hTERT-iDS (50PD) population. The primary hMSCs were shown to degrade β-galactosidase (blue coloration) when reaching 35PD, indicating an entry in a replicative senescence phase. The immortalized population bypassed the senescence-associated crisis and continued to proliferate.

E) Functional map of the iCasp9-ΔCD19 retrovector. The device drives the expression of a modified caspase 9 (iCaspas9) that can be induced toward dimerization thus leading to the activation of the apoptotic pathway. A 2A-like sequence between the iCaspase9 and CD19 gene ensures the cleavage of the CD19 surface marker from the iCaspase9, after transduction of the mRNA.

F) CD19 expression of the hTERT-iDS cell line. A homogenous population (>98%) could be sorted after CD19 labeling of the successfully transduced cells, resulting in the isolation of the hTERT-iDS population.

G) Functional assessment of the inducible death system implemented in the hTERT-iDS population. Prior to apoptosis activation, the majority (>93.6%) of the population was shown to be negative for Annexin-V and PI stainings (left panel). After activation of the system by an overnight exposure to the Chemical Inducer Dimerization (right panel), more than 99% of the cells were killed (positivity for Annexin-V or PI).

Figure 2: Osteogenic differentiation and killing assessment of M-SOD

A) Alizarin red staining of M-SOD cells after 3 weeks of culture in complete (CM) or osteogenic medium (OM). Only in presence of osteogenic medium, cells were able to deposit a mineralized matrix (red nodules). Size bar = 1cm

B) Gene expression levels of key osteoblastic genes after 14 days of culture in osteogenic medium. As compared to cells assessed prior to culture in OM (expanded cells), a significant upregulation of Alkaline Phosphatase (ALP), Bone Morphogenetic Protein 2 (BMP-2), Bone Sialoprotein, Osteocalcin (OC) and RunX2 (Run-related transcription factor 2) was observed. *, $p<0.05$.

C) Induction of death during osteogenic differentiation of the SOD cell line. Cells were induced toward death (+CID) after 1, 2, or 3 weeks of culture in osteogenic medium (OM). Following exposure to CID, more than 95% of the cells became Annexin-V or PI positive, independently of their differentiation state. ***, $p<0.001$.

Figure 3: Adipogenic/chondrogenic differentiation and killing assessment of M-SOD

A) Oil red-O staining of M-SOD after 3 weeks of culture in complete (CM) or adipogenic medium (AM). In presence of adipogenic medium, the M-SOD cell line was successfully differentiated into adipocytes as demonstrated by the positive staining of lipid droplets. Size bar = 200μm.

B) PPARγ gene expression level after 3 weeks of culture in complete or adipogenic medium. In adipogenic conditions, a significant overexpression of PPARγ mRNA level was detected (16.2 fold), thus confirming the capacity of M-SOD to differentiate into adipocytes. *, $p<0.05$

C) Induction of death during adipogenic differentiation of the M-SOD cell line. Cells were efficiently induced toward death (+CID) after 1, 2 or 3 weeks of differentiation (>95%) in adipogenic medium (AM). A high killing efficiency (>95%) was reached despite the differentiation of the cells. ***, $p<0.001$.

D) Alcian blue staining of M-SOD pellets after 2 weeks of culture in complete (CM) or chondrogenic medium (ChM). Both culture conditions did not lead to the generation of a rich cartilaginous matrix (blue staining). Size bar = 100µm

E) Collagen type II (Coll II) and type X (Coll 10) gene expression levels of M-SOD cells before (*expanded*) or after 2 weeks of pellet culture in chondrogenic medium (ChM). In chondrogenic conditions, only a significant overexpression of Coll 10 mRNA level was observed. ns = non-significant. *, $p<0.05$

Figure 4: Bone formation and Immunomodulatory capacities of M-SOD

A) In vivo bone formation of the M-SOD cell line. Cells successfully formed bone nodules (B) in contact with the ceramic granules (Ce), as demonstrated by Masson's trichrome staining (up) 8 weeks post *in vivo* implantation.Size bar = 50µm and 1 mm for the lower magnification inset.

B) Fluorescence microscopic analysis of bone formation of the M-SOD line. The eGFP signal (green) surrounding the bone nodules (B), demonstrates the contribution of M-SOD cells to the formed bone. Size bar = 50µm. The blue signal refers to cell nuclei staining (DAPI).

C) Immunomodulation assay (CFSE) of the M-SOD line. The percentage of proliferating CD4+ after 4 days of culture, with different ratios of M-SOD cells, +/- PHA activation.

D) Peaks of fluorescence in the activated CD4+, alone or in co-culture (1:5) with M-SOD cells, 4 days post PHA activation. In the absence of M-SOD, 5 peaks of fluorescence were observed corresponding to 4 division cycles.

Figure 5: Oncogenes expression levels and tumorigenicity assessment of M-SOD

A) Gene expression levels of key oncogenes in expanded primary hMSCs and M-SOD. The primary hMSCs and M-SOD expressed similar levels of p53, p21, Rb1 and c-Myc. *, $p<0.05$. f.d = fold difference.

B) Tumorigenicity assay of the M-SOD cell line. One million of M-SOD cells expressing luciferase were injected into the right flank of NOD/SCID mice (triplicate). Two days post cell injection, the luciferase system allows the localization of the M-SOD cells. After 8 days, the strength of the signal significantly decreased and was undetectable at day 16. More than 180 days post-injection, M-SOD cells remained undetectable so far demonstrating their non-tumorigenicity.

Figure 6: Schematic representations of the potential applications of the M-SOD line (See Text for more extensive description).

Supplemental data 1:

A) FACS sorting of the immortalized population using the eGFP expression of transduced cells. After sorting, the population homogeneously expressed the eGFP protein (>94%), indicating the successful isolation of the immortalized population.

B) Phase contrast microscopy image of the immortalized population (hTERT-MSCs, PD=65) after eGFP-based sorting. Cells display a typical spindle-shaped morphology. Size bar = 200µm.

C) Fluorescence microscopy image (UV light) assessing the homogenous eGFP expression in successfully transduced cells (hTERT-MSCs, PD=65). Size bar = 200µm.

Supplemental data 2: Alizarin red staining of the 24 clones after 3 weeks of culture in osteogenic medium. Among all the clones, the clone 20 was shown to be the most potent in depositing a mineralized matrix. All pictures were taken with a similar objective and showed a representative area of the well. Size bar = 500µm.

Supplemental Data 3: Multilineage differentiation assays of the primary hMSCs

A) Alizarin red staining of the primary hMSCs from whom M-SOD was derived after 3 weeks of culture in complete (CM) or osteogenic medium (OM). In the presence of osteogenic medium, cells were able to deposit a mineralized matrix (red nodules). Size bar = 1cm

B) Oil red-O staining of primary hMSCs after 3 weeks of culture in complete (CM) or adipogenic medium (AM). In the presence of adipogenic medium, the primary cells were successfully differentiated into adipocytes as demonstrated by the positive staining of lipid droplets. Size bar = 100μm.

C) Alcian blue staining of the primary hMSCs from whom M-SOD was derived. HMSCs pellets were stained after 2 weeks of culture in complete (CM) or chondrogenic medium (ChM). The absence of intense blue staining demonstrates the poor chondrogenic capacity of this donor. Size bar = 100μm.

Supplemental online video 1: Timelapse microscopy video (t = 5 hours) recording the apoptotic induction (hTERT-MSCs) by CID delivery (t = 0) to hTERT-iDS hMSCs. Within 5h, the large majority of the cells were successfully induced toward apoptosis. Size bar = 500 μm.

Table 1

	Primary hMSCs	hTERT-hMSCs			hTERT-iDS	
	PD=8	PD=75	PD=154	PD=170	Heterogenous PD=177	M-SOD PD >250
CD 44	+	+	+	+	+	+
CD 29	+	+	+	+	+	+
CD 73	+	+	+	+	+	+
CD 90	+	+	+	+	+	+
eGFP (hTERT)	-	+	+	+	+	+
CD 34	-	-	-	-	-	-
CD 45	-	-	-	-	-	-
CD 19 (death device)	-	-	-	-	+	+
CD 146	52.2%	**n.a**	67%	84%	+	+
Epcam	-	**n.a**	**n.a**	-	**n.a**	**n.a**
E-cadherin	-	**n.a**	**n.a**	-	**n.a**	**n.a**

+ positivity >90% **- negativity >90%** **n.a non assessed**

Figure 1

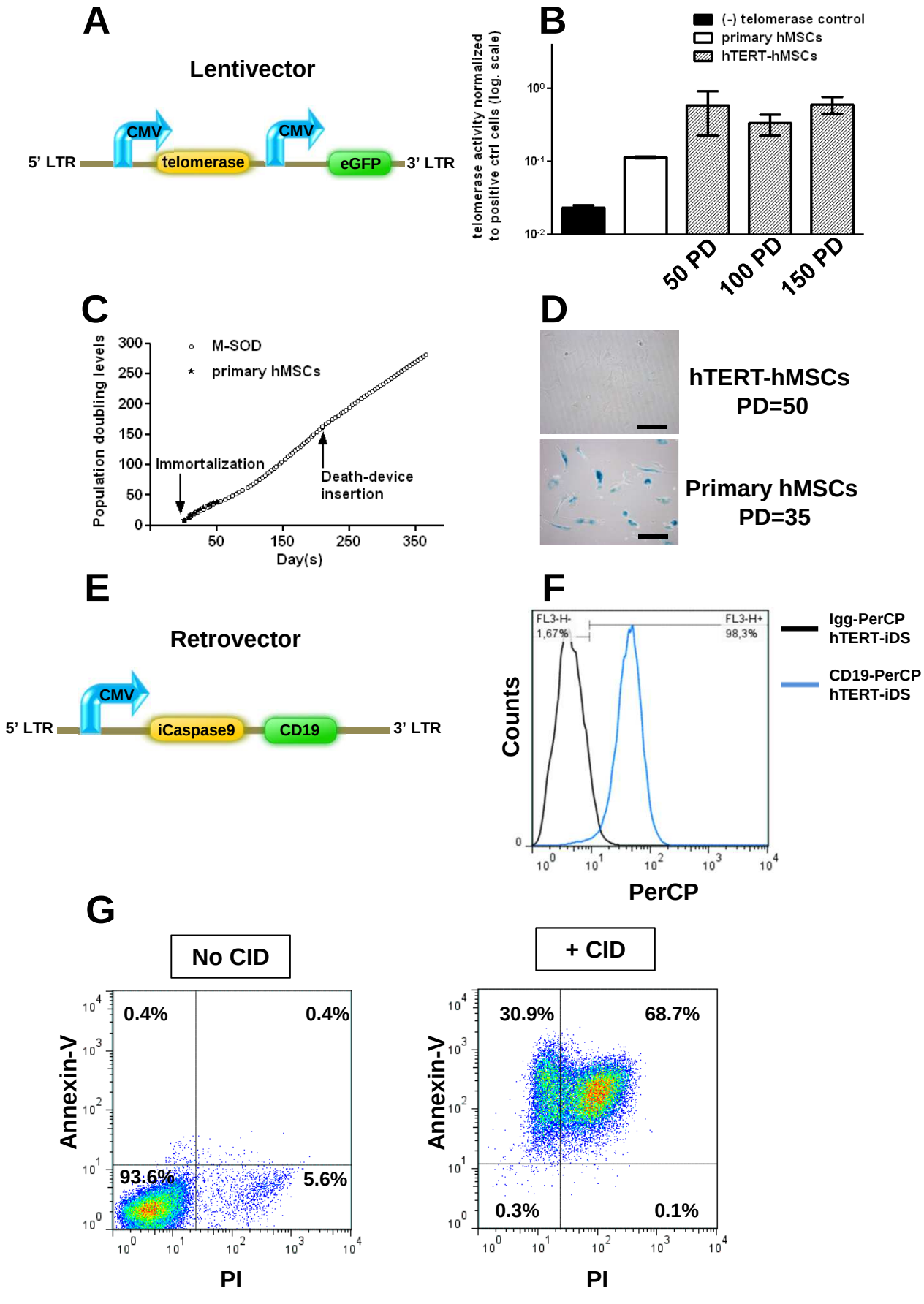

A
Lentivector
5' LTR
CMV
telomerase
CMV
eGFP
3' LTR
B
(-) telomerase control
primary hMSCs
hTERT-hMSCs
telomerase activity normalized to positive ctrl cells (log. scale)
50 PD
100 PD
150 PD
C
M-SOD
primary hMSCs
Population doubling levels
Immortalization
Death-device insertion
Day(s)
D
hTERT-hMSCs PD=50
Primary hMSCs PD=35
E
Retrovector
5' LTR
CMV
iCaspase9
CD19
3' LTR
F
FL3-H- 1,67%
FL3-H+ 98,3%
Igg-PerCP hTERT-iDS
CD19-PerCP hTERT-iDS
Counts
PerCP
G
No CID
+ CID
Annexin-V
PI
0.4%
0.4%
93.6%
5.6%
30.9%
68.7%
0.3%
0.1%

Figure 2

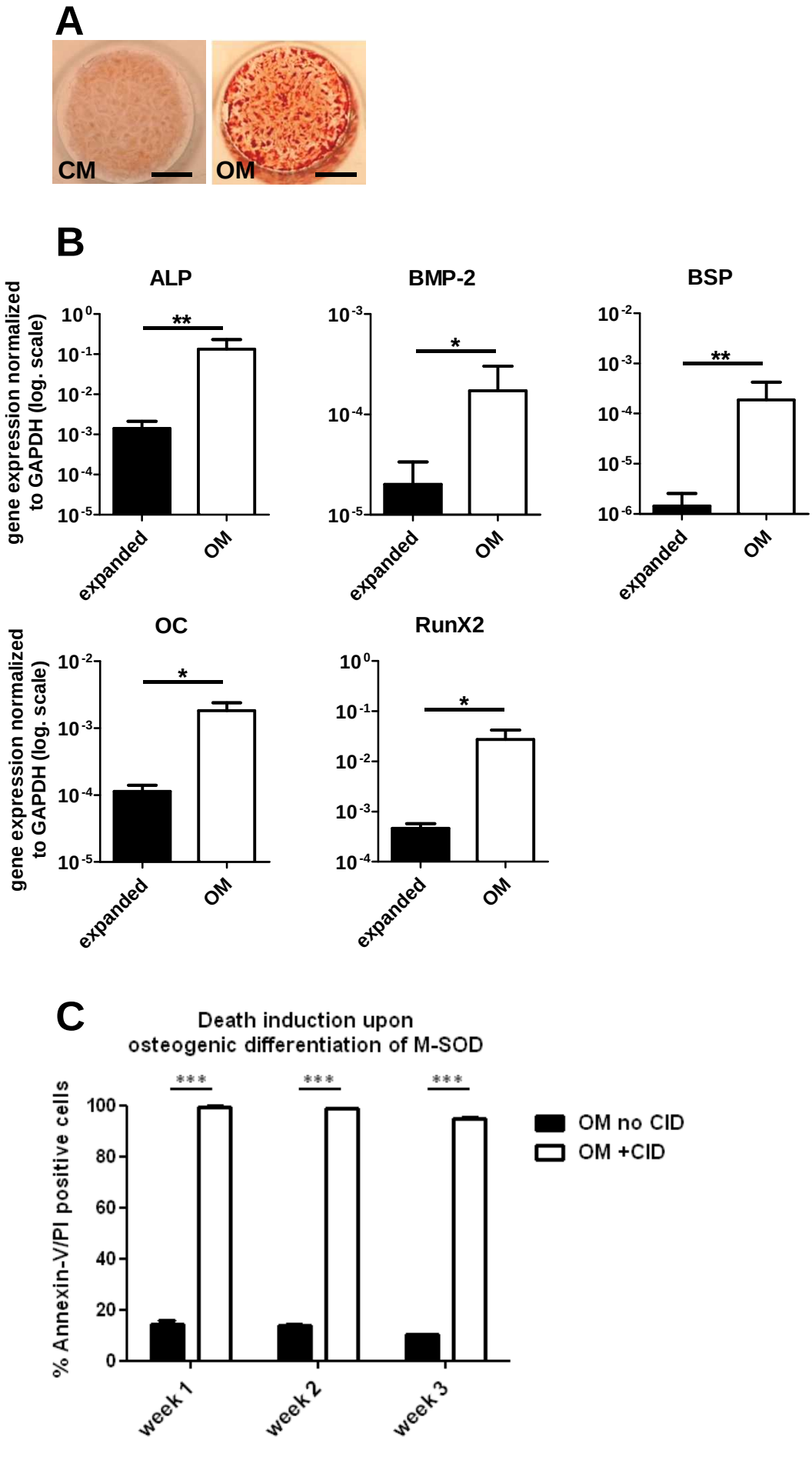

A
CM
OM
B
ALP
BMP-2
BSP
OC
RunX2
gene expression normalized to GAPDH (log. scale)
expanded
OM
C
Death induction upon osteogenic differentiation of M-SOD
% Annexin-V/PI positive cells
OM no CID
OM +CID
week 1
week 2
week 3

Figure 3

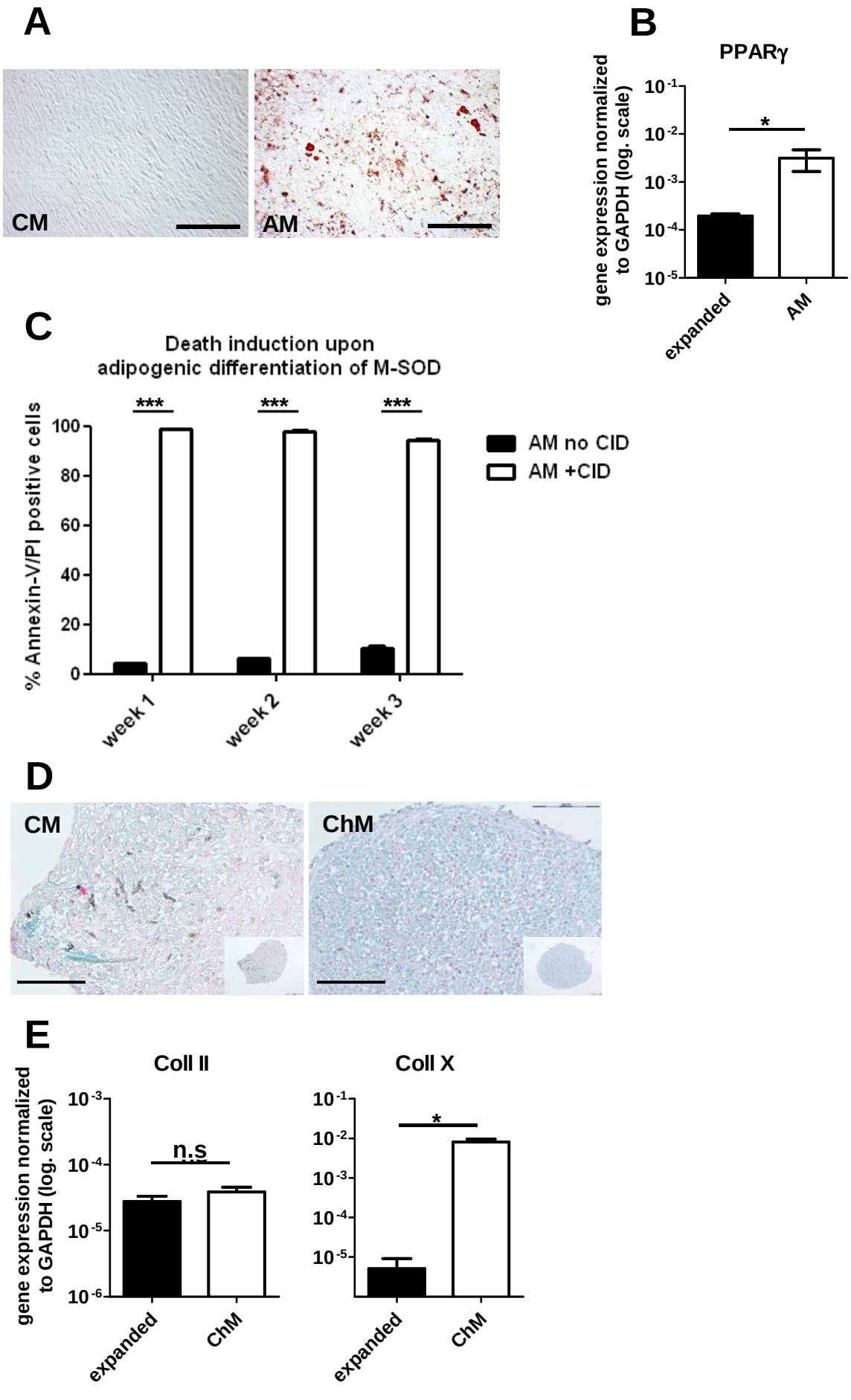

A
CM
AM
B
PPARγ
gene expression normalized to GAPDH (log. scale)
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
*
expanded
AM
C
Death induction upon adipogenic differentiation of M-SOD
% Annexin-V/PI positive cells
100
80
60
40
20
0
***
***
***
AM no CID
AM +CID
week 1
week 2
week 3
D
CM
ChM
E
Coll II
Coll X
gene expression normalized to GAPDH (log. scale)
10^{-3}
10^{-4}
10^{-5}
10^{-6}
n.s
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
*
expanded
ChM
expanded
ChM

Figure 4

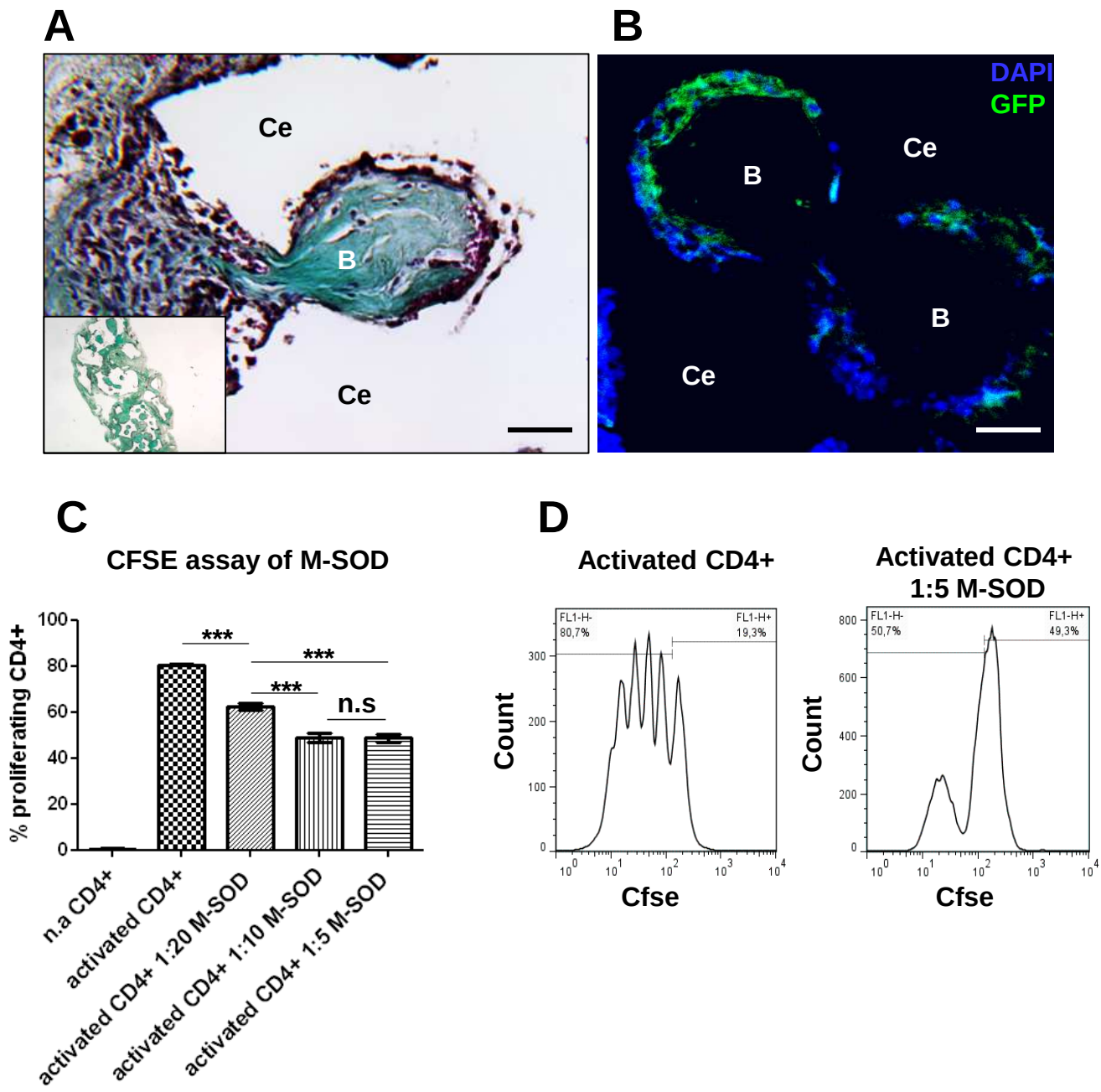

A
Ce
B
Ce
B
DAPI
GFP
Ce
B
B
Ce
C
CFSE assay of M-SOD
% proliferating CD4+
100
80
60
40
20
0
***
***
***
n.s
n.a CD4+
activated CD4+
activated CD4+ 1:20 M-SOD
activated CD4+ 1:10 M-SOD
activated CD4+ 1:5 M-SOD
D
Activated CD4+
FL1-H-
80,7%
FL1-H+
19,3%
Count
Cfse
Activated CD4+
1:5 M-SOD
FL1-H-
50,7%
FL1-H+
49,3%
Count
Cfse

Figure 5

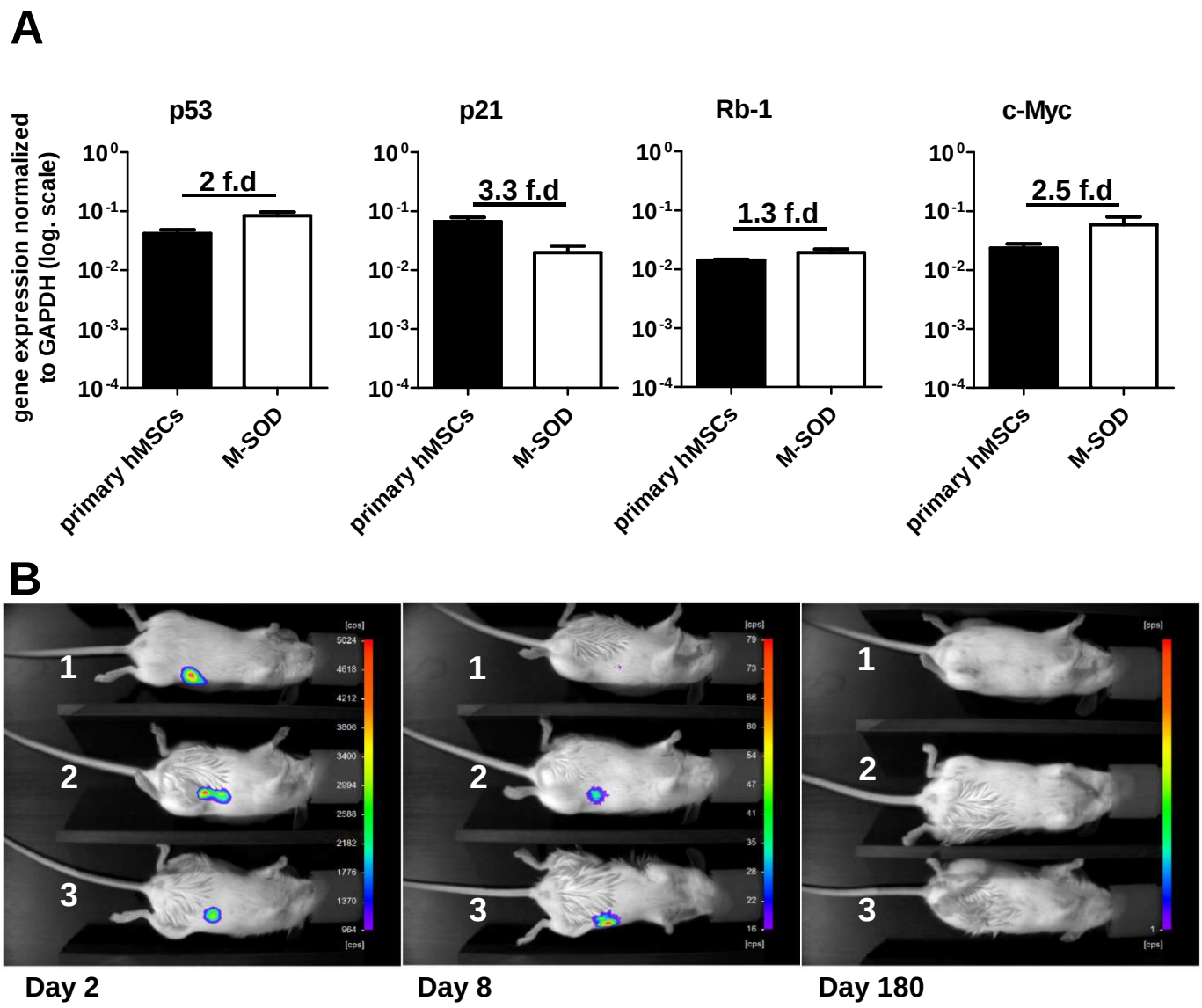
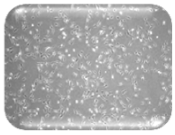
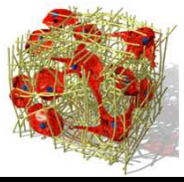

A
p53
p21
Rb-1
c-Myc
gene expression normalized to GAPDH (log. scale)
2 f.d
3.3 f.d
1.3 f.d
2.5 f.d
primary hMSCs
M-SOD
B
1
2
3
Day 2
Day 8
Day 180

Figure 6



Experimental tool to study hMSCs

- Steady proliferation
- Standardized differentiation
- Temporally controlled cell elimination (co-culture system)



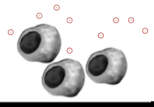
Engineering of devitalizable matrices

- Efficient killing
- Matrix integrity preservation

M-SOD

Cellular therapy

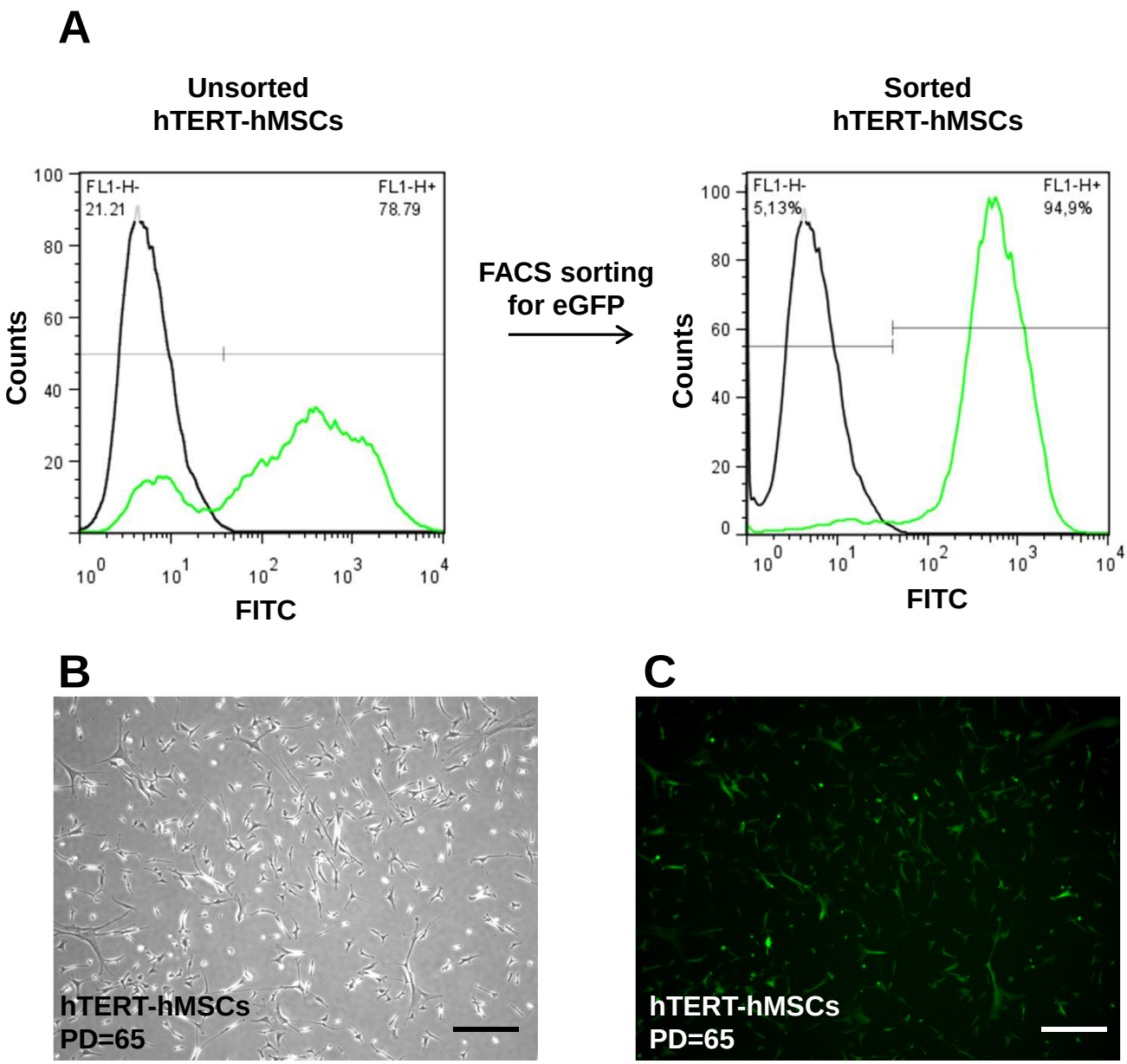
- Standardization
- High cell characterization
- Pre-requisites for safety



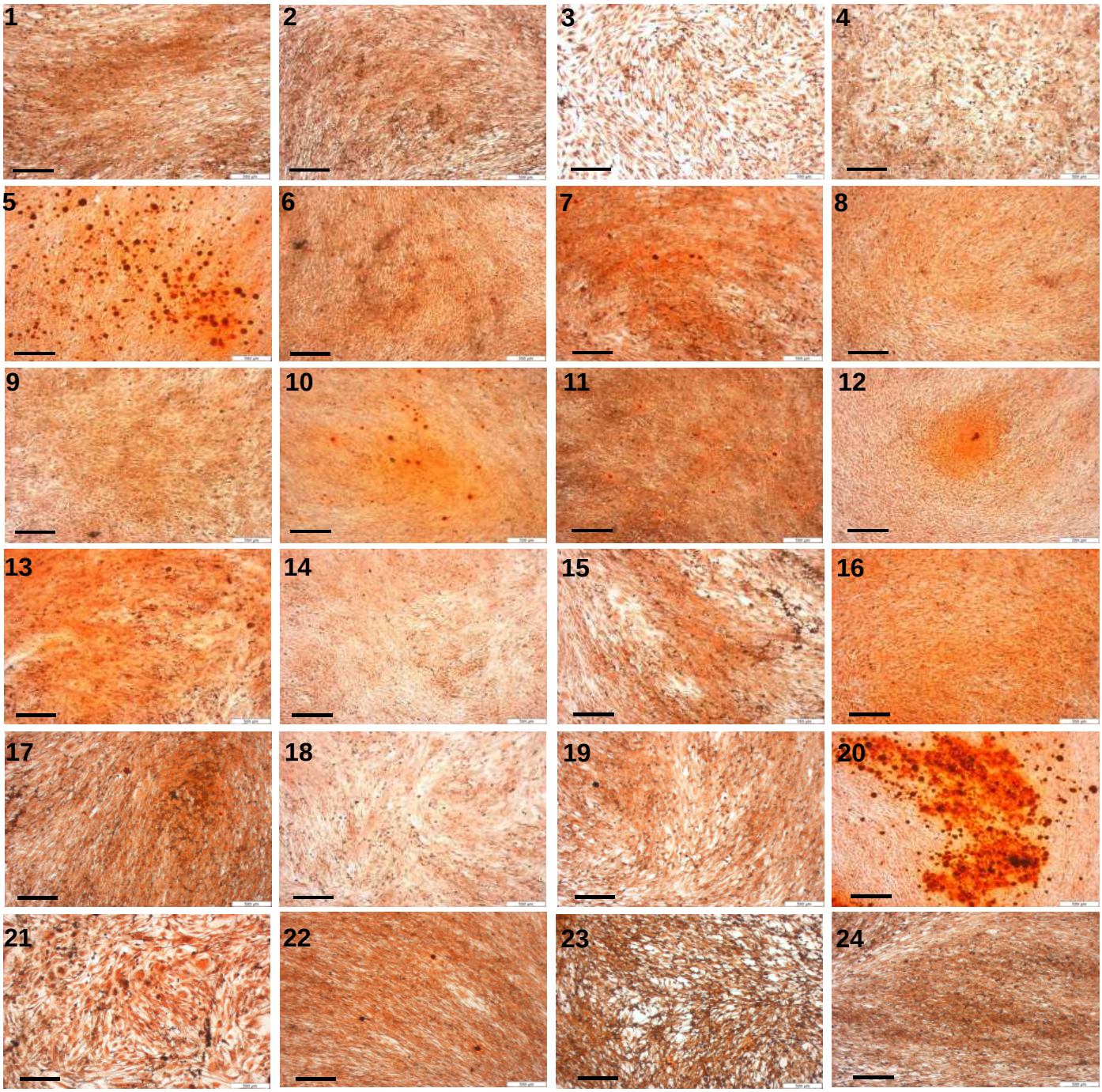
Cell-based drug delivery system

- Cell customization (additional transductions)
- Temporally controlled factor delivery

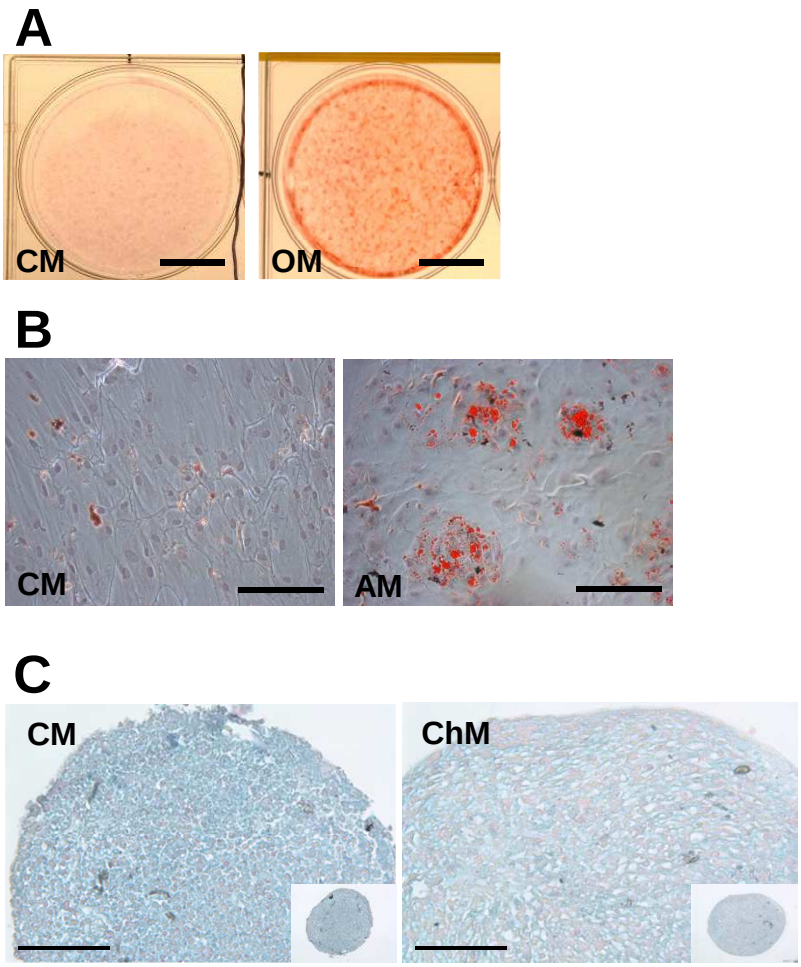
Supplementary data 1



Supplementary data 2



Supplementary data 3



Chapter 3

“Tissue decellularization by activation of programmed cell death”

Enclosed is the pdf file of the paper published in Biomaterials, 2013 Aug;34(26):6099-108

Biomaterials 34 (2013) 6099–6108

Contents lists available at SciVerse ScienceDirect

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Leading opinion

Tissue decellularization by activation of programmed cell death

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ARTICLE INFO

Article history:
Received 19 April 2013
Accepted 27 April 2013
Available online 27 May 2013

Keywords:
Decellularization
Apoptosis
Bioreactor
Regenerative medicine
Extra-cellular matrix

ABSTRACT

Decellularized tissues, native or engineered, are receiving increasing interest in the field of regenerative medicine as scaffolds or implants for tissue and organ repair. The approach, which offers the opportunity to deliver *off-the-shelf* bioactive materials without immuno-matching requirements, is based on the rationale that extracellular matrix (ECM)-presented cues can be potently instructive towards regeneration. However, existing decellularization protocols typically result in damage to the source ECM and do not allow the controlled preservation of its structural, biochemical and/or biomechanical features. Here we propose the deliberate activation of programmed cell death as a method to selectively target the cellular component of a tissue and thereby to preserve the integrity of the decellularized ECM. In the case of engineered tissues, the approach could be complemented by the use of (i) an immortalized cell line, engineered to undergo apoptosis upon exposure to a chemical inducer, and (ii) a perfusion bioreactor system, supporting efficient removal of cellular material. The combination of these tools may lead to the streamlined development of more appropriate materials, based on engineered and decellularized ECM and including a customized set of signals specifically designed to activate endogenous regenerative processes.

1. Introduction

The extracellular matrix (ECM) is a combination of structural and functional proteins, proteoglycans, lipids and crystals that has a unique composition and physical properties for every tissue and organ in the body. Acting as a reservoir for morphogens while providing mechanical support for resident cells, ECM participates in cell communication as well as in defining the shape and stability of tissues [5]. ECM cues have been demonstrated to specifically promote cell recruitment, adhesion, migration, proliferation and differentiation in a way that reflects the functional needs and biological identity of tissues [6]. Such instructive elements may retain at least in part their functionality even in the absence of the living cellular component.

Based on this rationale, decellularized ECM has received increased attention in the field of regenerative medicine as an *off-the-shelf* and immune-compatible alternative to living grafts for tissue and organ repair (Fig. 1). Decellularized ECM is expected to induce regenerative processes not only through specific "organomorphic" structures [8], but also by the physiological presentation of different cocktails of regulatory molecules in a mechanically suitable environment. The instructive scaffold materials derived from decellularized ECM could be activated by living cells prior to implantation, with the assumption that ECM is capable of directing the differentiation fate of the seeded cells [9–12]. In an even more attractive paradigm, the decellularized ECM could be directly used to instruct resident cells towards endogenous tissue repair by leveraging principles of morphogenesis. Starting from decellularized bone as a prototype ECM graft [13], the field has received convincing proof-of-principle evidences of the latter approach for epithelial [14], musculoskeletal [15] and vascular [16] tissue regeneration, as well as for the engineering of myocardial [17], pulmonary [4], renal [18,19] and pancreatic implants [20]. More recently, thanks to the progress in guiding cell differentiation towards specific lineages, *in vitro*-engineered tissues are also being considered as a substrate for decellularization. This approach opens the perspective to the generation of large quantities of standardized, customized grafts.

A variety of chemical, enzymatic and physical procedures have been developed to eliminate the cellular component of both native and engineered tissues while minimally disrupting the ECM (Box 1). Protocols described in literature tend to combine several of these principal methods in order to increase the efficiency of decellularization and at the same time reduce damage to the ECM by using less destructive conditions.

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http://dx.doi.org/10.1016/j.biomaterials.2013.04.058

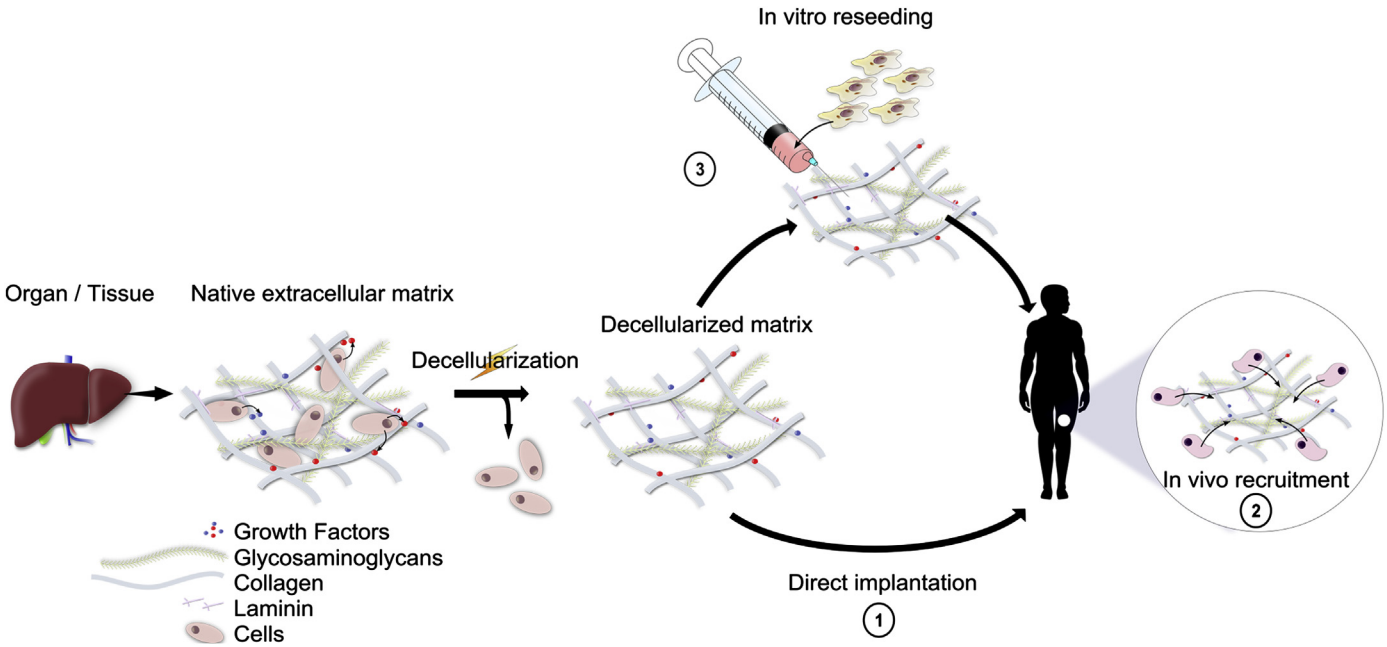


Fig. 1. Concept of tissue decellularization. Cell-free tissue can be generated by decellularization of native or engineered tissue. The resulting ECM can be directly transplanted into a patient (1), entirely relying on the capacity to instruct resident cells towards endogenous tissue repair (2). Alternatively, prior to implantation the ECM can be seeded with cells that "prime" the material (e.g., to enhance its remodeling or vascularization) and/or "get primed" toward a specific function (e.g., to proliferate or differentiate) (3). The latter implants could induce regeneration by the combined action of the seeded and recruited cells.

Box 1
Overview of existing decellularization procedures. A more comprehensive analysis can be found in recent reviews [6,22,23].

Acids and bases [1] react with and denature proteins, solubilize cell components and alter nucleic acids, thus bursting cells. They are not selective and hence alter also ECM components, especially collagens, glycosaminoglycans (GAG) and growth factors.

Hypertonic and hypotonic solutions [2] disrupt cells through osmotic shock and also interfere with DNA-protein interactions. They have a relative efficiency and do not allow for an effective removal of cellular residues.

Detergents [3] may be ionic, non-ionic or a combination of both. They disrupt DNA-protein, lipid and lipid–protein interactions and may denature proteins. Additionally to their action on cells, they also damage the ECM ultrastructure, leading to a loss of collagens, growth factors and GAG.

Enzymes [4] can be used to target the remnant nucleic acids after cell rupture or the peptide bonds which anchor the cells to the ECM. They tend to remain in significant quantities in the tissue and may provoke an additional immune response. Prolonged exposition can also result in removal of collagens, laminin, fibronectin, elastin and GAG, as well as destruction of the ECM ultrastructure.

Physical methods [7] are widely used for the decellularization of tissues. The most common ones consist of repeated freeze and thaw cycles. Cells burst due to the formation of intra- and extra-cellular ice crystals, which lead to drastic changes in ion concentrations and mechanical destruction of the cell membrane. The ice crystals also damage the ultrastructure and proteins contained in the ECM and can inactivate some growth factors (e.g., Vascular Endothelial Growth Factor (VEGF)).

All of the above mentioned methods, which have been subject of several review assays [6,21–23], can reach variable degrees of decellularization efficiency, but some problems remain common to all. First, all existing techniques rely on cell lysis. The resulting cell debris can then freely adsorb to the remaining matrix, leading to a paradoxical increase in immunogenicity [24]. Second, existing techniques have been demonstrated to alter the ECM, leading to the degradation of some of its components [6].

In principle, maintaining the integrity of the ECM following decellularization could support a more predictable, reproducible and effective clinical use of the resulting material. Moreover, from a scientific standpoint, preservation of the ECM would be instrumental to possibly identify the role of specific molecules and their organization in eliciting tissue repair processes. However, the typical procedures used necessarily imply an impairment of the ECM integrity. Aiming at avoiding the side effects inherent to current strategies, we propose here an alternative approach to tissue decellularization based on the controlled activation of programmed cell death.

2. Processes of cell death

Cell death can occur by either necrosis or apoptosis. During necrosis, the cell membrane rapidly becomes permeable, leading to the leakage of the intra-cellular content. Necrosis is a non-lysosomal, uncontrolled process typically caused by external events and signals [25], associated with distinct morphological patterns (Fig. 2). Since necrosis involves the early loss of the membrane integrity, it induces a massive inflammation linked to the leakage of cellular content [26].

Instead, apoptosis is a genetically determined process, consequently referred to as the "programmed cell death", leading to the suicide of the cells [27,28]. Apoptosis typically occurs during tissue and organ development but may also act as a homeostatic mechanism or as a means to eliminate infected or tumorigenic cells [29]. Towards the induction of apoptosis for tissue decellularization, it is necessary to distinguish two different pathways, namely the

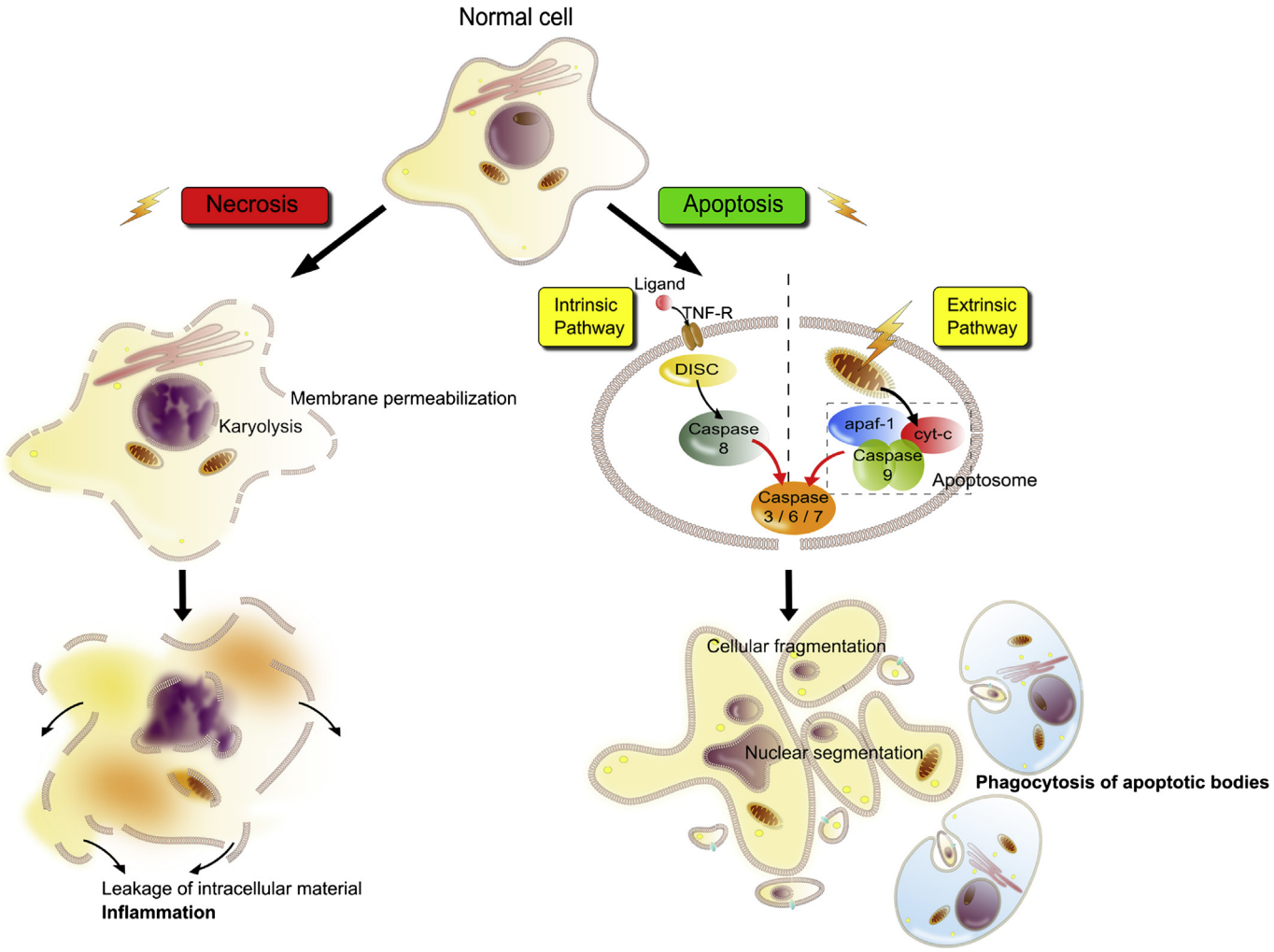


Fig. 2. Processes of cell death. Cell death occurs through either necrosis or apoptosis. Necrosis implies permeabilization of the cell membrane and consequently the leakage of the intra-cellular content, potentially inducing massive inflammation. Instead, the activation of the apoptotic program, through either the intrinsic or extrinsic pathway, involves a cascade of molecular events that results in cellular condensation and disruption into small apoptotic bodies. During the process, the cellular content is degraded and strictly kept within the cell membrane, avoiding an inflammatory reaction. Abbreviations: TNFR = tumor necrosis factor receptor; apaf-1 = apoptotic protease activating factor 1; cyt-c = cytochrome-c.

intrinsic or extrinsic ones. While the two pathways differ in their initial activation, they converge in the final cell suicide execution (Fig. 2). The molecular mechanisms driving both apoptotic pathways are highly complex and not fully understood, but can be shortly described as follows.

The intrinsic pathway, triggered upon stress stimuli (e.g. change of environmental conditions), is initiated by the permeabilization of the mitochondrial outer membrane, resulting in the release of the cytochrome-c (cyt-c) into the cytosol [30]. This protein forms a complex called apoptosome, together with the Apoptotic protease activating factor 1 (Apaf-1) and caspase-9. The formation of this complex turns on the initiator caspase-9, which in turn activates the effectors caspase-3, 6, and 7 [31–33]. Due to the essential role of the mitochondria in this process, the intrinsic pathway is also referred to as the "mitochondrial pathway".

The extrinsic pathway is triggered by external signals binding to specific cell surface death receptors (DR) of the tumor necrosis factor family (TNF, Table 1) [34,35]. Such binding results in the formation of a death inducing signaling complex (DISC), leading to the transduction of the signal within the cytosol [36]. Following caspase-8 dimerization, the same effector caspases involved in the intrinsic route are activated.

After initiation of the suicide program by either the intrinsic or extrinsic pathway and following the activation of the effector caspases, different endonucleases and proteases respectively lead to DNA fragmentation and the degradation of cytoskeletal and nuclear proteins [28]. As a consequence, cytomorphological changes are

Table 1
Death receptors of the TNF superfamily and their cognate ligands. Abbreviations: TNFR1 = tumor necrosis factor receptor 1; TNFα = Tumor necrosis factor α; Apo-3 = Apoptosis antigen 3; TRAIL = TNF-related apoptosis inducible ligand; TRAILR = TNF-related apoptosis inducible ligand receptor; NGF = nerve growth factor; NGFR = nerve growth factor receptor; EDA = ectodysplasin A; EDAR = ectodysplasin A receptor [38,39,81,82].

Receptor	Cognate ligand
TNFR1 (DR-1)	TNFα
Fas/CD95 (DR-2)	FasL
Apo-3 (DR-3)	TL1A
TRAILR (DR-4 and DR-5)	TRAIL
DR-6	Unknown
NGFR	NGF
EDAR	EDA

observed like the chromatin and cytosol condensation, associated with a global cell shrinkage [28]. As a final step, cells are disrupted into small apoptotic bodies that are then phagocytosed by immune cells [37]. This common end stage cellular morphology of intact apoptotic bodies is particularly interesting when considering the decellularization of tissues and matrices, as described below.

3. Apoptosis, the holy grail for tissue decellularization?

Existing decellularization methods require a tradeoff between efficient cellular removal and ECM preservation, as increasing the treatment severity for a more complete decellularization fatally results in an enhanced ECM disruption. As a result, current decellularization protocols rather aim at minimizing effects on the ECM rather than avoiding them [6]. Here we propose an alternative approach based on the exploitation of programmed cell death. The apoptotic pathways can be deliberately activated through a clean and controlled process by the delivery of appropriate signals. Importantly, this strategy of decellularization would selectively target the cell component of a tissue, in a way which remains decoupled from compromising the ECM integrity.

A closer analysis of the mechanism of apoptosis identifies key features that make it attractive in view of a decellularization procedure. During apoptosis, the cell membrane undergoes structural changes that lead to the loss of cell contact with the ECM [38]. This combined with the reduced size of apoptotic bodies (0.5–2 μm), cell removal with virtually no structural or functional changes to the tissue matrix may be achievable. Moreover, during the whole apoptotic process, the cellular content is kept strictly within the cell membrane and the apoptotic bodies [39]. The immunogenic cellular constituents do not leak into the surrounding matrix [40–42], thus preventing an unnecessary inflammatory reaction. This is in contrast to existing decellularization techniques, inducing a necrotic cell-death, cell bursting and the release of immunogenic cellular material within the neighboring environment [24]. Without appropriate rinses, such material may generate a high immune-reaction following implantation and subsequently lead to the rejection of the graft.

A possible additional advantage of ECM decellularization by induction of apoptosis is related to the association between the activation of effector caspases [3,6,7] and the release of Prostaglandin E_2 (PGE_2). This process has been recently reported to be involved in tissue regeneration by stimulating proliferation of neighboring progenitor cells [43]. Thus, decellularizing a tissue through the apoptotic pathway may contribute to prime the matrix towards regeneration programs through the release of key paracrine signals.

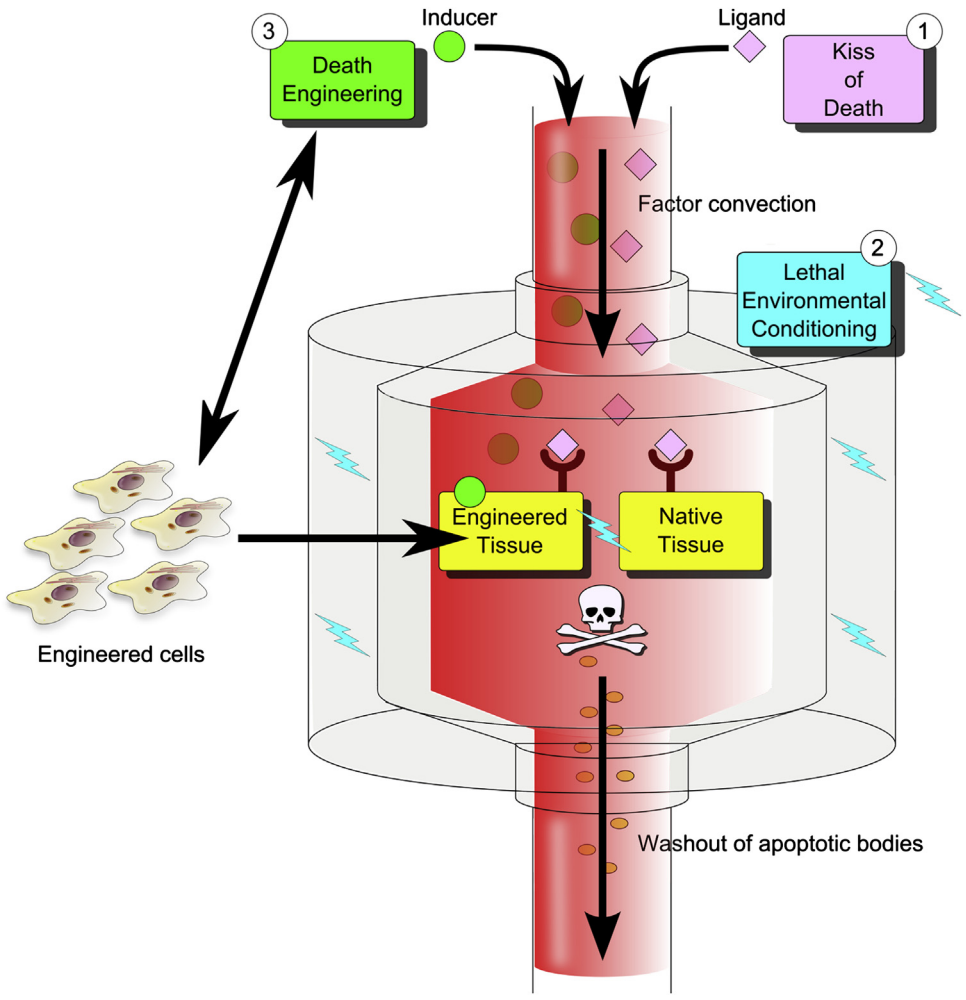


Fig. 3. Tissue decellularization by apoptosis induction within 3D perfusion bioreactor. Three different approaches are proposed in order to decellularize a tissue by apoptosis-induction; *Kiss-of-death* (1), *Lethal Environmental Conditioning* (2), *Death-engineering* (3). The decellularization procedure within a 3D perfusion bioreactor system increases the convection of the apoptotic inducer, establishes controlled environmental conditions and facilitates the washout of apoptotic bodies. In particular, when considering the decellularization of engineered tissues, this system could be the basis for a streamlined process to generate an ECM and subsequently induce apoptosis within a single, closed device.

Apoptosis is also a physiological process during embryogenesis, normal tissue development and immunological events. Induction of apoptosis, as the predominant pathway to cell death, thus has the potential to mimic natural developmental steps in tissue formation and remodeling [44,45]. Hence, the proposed strategy appears to be in compliance with the recently described concept of "developmental engineering" [46], namely the recapitulation of developmental processes for the engineering of regeneration.

4. Apoptosis induction strategies

The method used to implement the apoptotic concept for ECM decellularization will critically determine the killing efficiency and overall success of the strategy. Here we propose different approaches, which notably differ according to the type of pathway (i.e., intrinsic vs. extrinsic) to be activated.

4.1. Kiss-of-death

This strategy entirely relies on the extrinsic pathway activation through the delivery of specific ligands that bind their corresponding death receptors (DRs) of the TNF superfamily (Table 1). As the DRs are ubiquitously expressed [47], the activation of the extrinsic apoptotic pathway can be considered for the decellularization of virtually any tissue/organ. Nevertheless, the choice of the ligand, and consequently the type of DR to target has to be carefully considered. While some ligands are known to specifically activate the programmed cell death, others (DR-1, DR-3, DR-6 and EDAR) may have also anti-apoptotic effects, leading to the activation of survival signals [35]. Furthermore, each cell type might have a different sensitivity to each ligand, since a differential expression of the DRs is observed from tissue to tissue. Some cells may also be more prone than others to escape apoptosis through the survival pathway. For instance, TRAIL was shown to induce apoptosis in cancer cells, but not in normal cells [48,49]. Therefore, when elaborating a decellularization strategy, the type of DR to target should be selected according to the specific cellular system.

A certain number of pro-apoptotic ligands were already reported to successfully induce apoptosis in a variety of cell types. Human bone marrow-derived mesenchymal stromal cells (hMSC) were shown to be sensitive to FasL, in both an undifferentiated and differentiated status [50]. FasL was also shown to induce apoptosis of cardiomyocytes [51] and epidermal cells [52]. Moreover, it is abundantly expressed in a variety of organs, such as the heart, kidney, pancreas and the liver [53]. The delivery of TNFα was described as an efficient inducer in lung [54] and intestinal epithelial cells [55]. This factor also promotes chondrocyte [56] and renal endothelial cell apoptosis [57].

This non-exhaustive list of references demonstrates the diversity of the studies reporting the successful use of DR ligands as apoptotic inducers. However, none of them aimed at optimizing the observed killing, which may require screening not only for the appropriate ligand, but also for a suitable concentration and time of induction. One possible optimization strategy could rely on the combination of ligands, to generate a "customized death cocktail". This can be used to obtain synergistic effects, as for example one ligand would prime cells to increase their sensitivity to a second one. It was thus shown that treating lung fibroblast and hepatocytes with TNFα sensitizes them to FasL induced apoptosis [58,59].

A potential limit of the *Kiss-of-death* strategy possibly involves the inflammatory effect of some ligands. A certain quantity of those factors may remain entrapped in the ECM, requiring additional rinsing for their removal from the graft. Furthermore, depending on the required concentrations of the selected ligands, the process may become costly.

4.2. Lethal-environmental-conditioning

Upon environmental stress, cells can naturally undergo apoptosis by activation of the intrinsic "mitochondrial" pathway. An apoptotic response could thus be induced by modulating

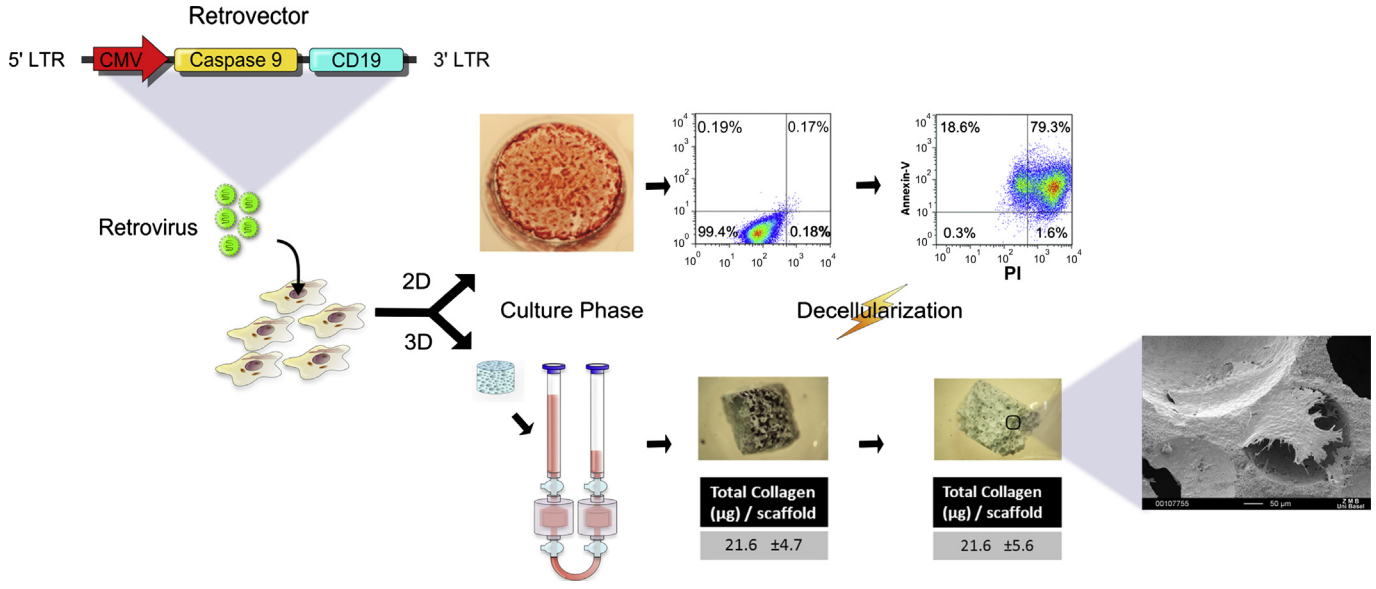
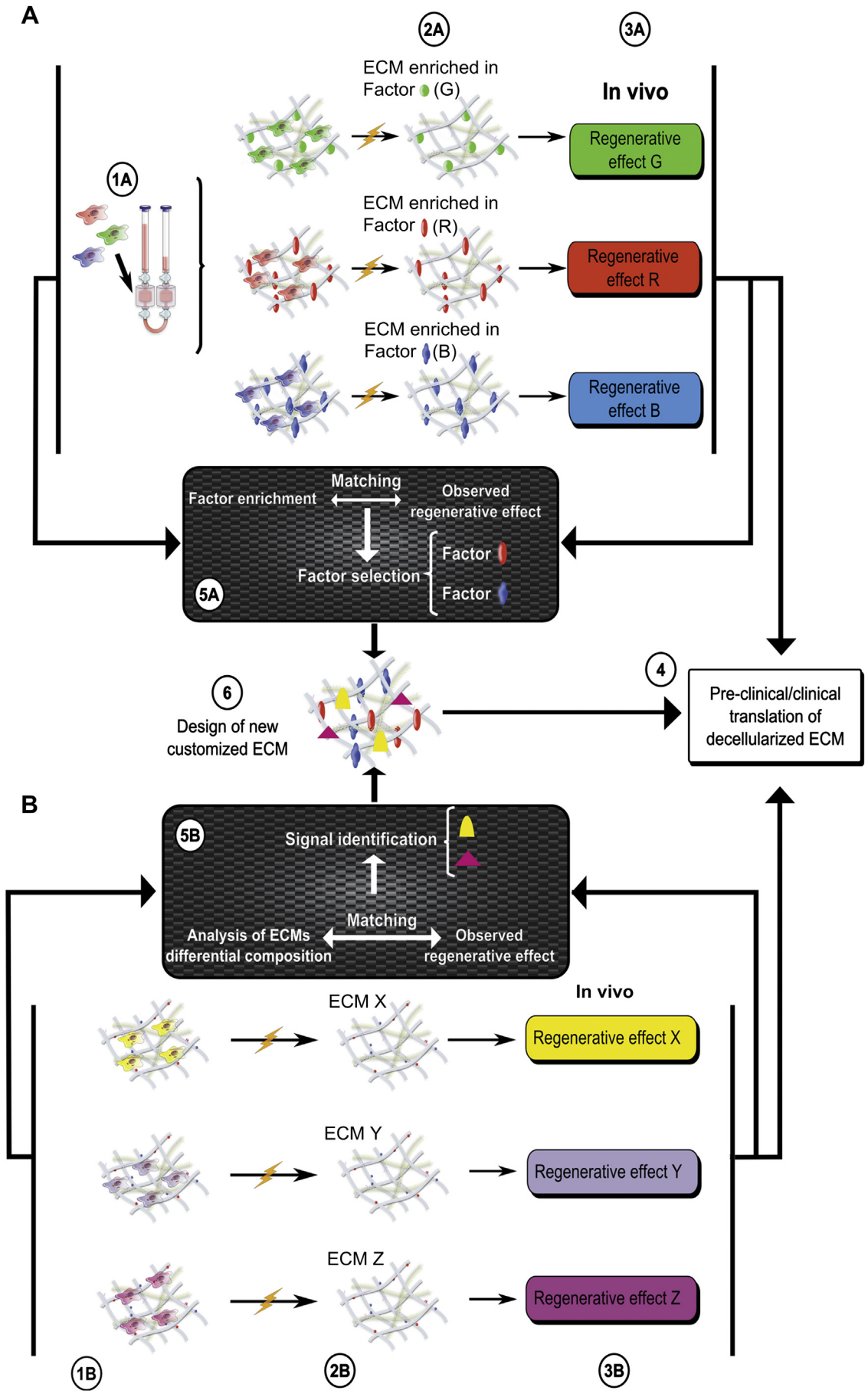


Fig. 4. Generation of ECM-decorated materials by apoptosis-driven decellularization. A hMSC cell line was generated and transduced with an inducible caspase 9 gene (iCaspase 9) [71]. Cells were shown to deposit a mineralized ECM (Alizarin-red staining) in Petri dishes (2D) while being efficiently inducible toward apoptosis (>95% positivity for Annexin-V and/or PI). After perfusion-mediated seeding on porous ceramic scaffolds (3D), the cell line was capable to adhere, colonize the scaffold (MTT staining) and deposit a collagen-rich ECM. The apoptotic decellularization performed within the bioreactor system led to the generation of an ECM-decorated material without a measurable reduction of the total collagen content. As a comparison, the freeze&thaw method applied to the same constructs resulted in a collagen loss of 74% (final content: 5.61 ± 1.06 µg/scaffold). These findings provide a proof-of-principle for the decellularization of ECM produced by osteogenic cells following the described apoptotic *Death-engineering* strategy, in a perfusion bioreactor setup.


A
2A
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ECM enriched in Factor (R)
Regenerative effect R
ECM enriched in Factor (B)
Regenerative effect B
Factor enrichment
Matching
Observed regenerative effect
Factor selection
Factor
Factor
5A
6
Design of new customized ECM
4
Pre-clinical/clinical translation of decellularized ECM
B
5B
Signal identification
Matching
Analysis of ECMs differential composition
Observed regenerative effect
In vivo
ECM X
Regenerative effect X
ECM Y
Regenerative effect Y
ECM Z
Regenerative effect Z
1B
2B
3B

environmental factors, including temperature, pH, as well as CO_2/O_2, nitric oxide (NO), and H_2O_2 content. As opposed to the physical freeze & thaw method, leading to a necrotic response, induction of apoptosis by temperature changes requires low variations, in either hyperthermic [60] or hypothermic [61] ranges. Ideally, temperature fluctuations from 10 °C to 45 °C are advised, whereby the effect on the matrix will also depend on the incubation time. NO has been described as a potent inducer of mitochondrial apoptosis [62], especially for cardiomyocytes [63], pancreatic cells [64] and chondrocytes [65]. The use of hypoxic conditions was also reported to activate the apoptotic program in pancreatic cells [66] or cardiomyocytes [67].

The advantages of the *lethal-environmental-conditioning* strategy rely on the minimal cost and simplicity of the setup required to modulate and control the operating conditions. However, an efficient killing might only be obtained after an extensive and possibly multi-parametric screening for optimal conditions.

4.3. Death-engineering

This strategy relies on the activation of any of the two apoptotic pathways by the use of a genetic approach. Since it requires the genetic engineering of cells prior to the generation of a tissue, this strategy relates mainly to the decellularization of engineered grafts. Apoptosis activation could in principle be achieved by modulating the expression level of key genes involved in the pathway. Nevertheless, since apoptosis implies phosphorylation and dimerization of specific molecular players, it cannot be triggered by simply overexpressing or silencing key genes of the transduction pathway.

One possibility to engineer cells to death consists in overexpressing death-receptors at the cell surface. This was shown to enhance cell sensitivity to the death-inducing ligand [68] and could thus be used in conjunction with the *Kiss-of-death* strategy.

An alternative option relies on the implementation of a toxic transgene (e.g. apoptin, lectin), whose expression is under strict control. In this regard, the use of a tight and inducible expression system is a requirement in order to activate cell-suicide post-tissue generation and to avoid an excessive “leakiness” that may also result in premature cell-death. The stable integration of such a genetic construct is also critical as existing transient expression systems were shown to persist only from days to a few weeks within the cells [69]. Considering modified cells would be induced towards death after the synthesis of a tissue, which implicates an extensive culture time, transient systems may result in a poor killing due to the non-persistence of the genetic construct.

As a suitable genetic device addressing the above mentioned requirements, an elegant inducible system was described [70] and shown to lead to a high killing efficiency in transduced cells. This device is based on the constitutive expression of a modified caspase 9, whose dimerization can be activated through the delivery of a clinically approved inducer. This genetic approach was originally developed to improve the safety of cell-based therapy, but could also be used to induce the decellularization of a tissue. The efficiency of killing was already demonstrated in primary and differentiated hMSC [71]. The advantage of this system relies in the absence of leakiness and the downstream activation of the apoptotic pathway, thus avoiding activation of the survival pathway.

The three aforementioned strategies (*Kiss-of-death, lethal environmental conditioning and death-engineering*) differ in protocol but are fully compatible and their combination may in fact synergize the final cell killing efficiency.

5. Tissue/organ decellularization within perfusion systems

Devitalization can be considered as the first step of a decellularization procedure, ultimately aiming not only at killing, but also at removing the cellular fraction. Typical methods to eliminate the cell debris are based on extensive rinsing but risk to either lead to mechanical disruption of the tissue or to a non-efficient removal of the immunogenic material. Following the concepts of whole organ perfusion elegantly developed in the recent years [5,17], here we propose the use of perfusion bioreactor systems combined with induction of apoptosis in order to achieve an efficient and controlled tissue decellularization (Fig. 3).

The use of a bioreactor system would allow for a superior control of the process parameters, such as temperature or gas content, which would be directly relevant for the *lethal-environmental-conditioning* strategy. Moreover, the perfusion may enhance the killing efficiency by increasing the convection of the pro-apoptotic factors throughout the graft, especially while considering the *Kiss-of-death* or *Death engineering* strategies (Fig. 3). The use of perfusion systems would also play a critical role in the efficient and controlled removal of the previously killed cellular component. Controlling the flow patterns and associated induced shear may be of interest during the washing step in order to eliminate the apoptotic bodies from the ECM as they form, thus allowing for a non-invasive, yet efficient and standardized wash-out of any cellular material.

The use of perfusion-based bioreactor systems, forcing a cell suspension or culture medium directly through the pores of a scaffold material, was previously shown to support the engineering of tissues with superior properties than typical static cultures [72–74]. Thus, one could envision a streamlined manufacturing process for *off-the-shelf* decellularized grafts, whereby the same perfusion system would be used first to develop the tissue and subsequently to decellularize the deposited ECM. Additional features and benefits of such paradigm, derived from other biotechnology settings, would include automation, standardization, control and possibly cost-effectiveness of the process implementation.

6. Tissues engineered to death: a proof of principle

In the context of bone regeneration, one attractive strategy is to generate decellularized grafts with osteo-inductive properties by decoration of materials with a cell-laid ECM. In contrast to the delivery of over-doses of single morphogens (e.g., defined bone

Fig. 5. Alternative pathways towards the design of customized, decellularized ECM (A) Cells can be engineered (1A) to secrete specific factors/morphogens. Following 3D culture, possibly in a bioreactor system and subsequent apoptosis-driven decellularization, the produced tissues are enriched in specific morphogens (2A). The differences in composition are expected to trigger specific regenerative effects *in vivo* (3A) that could result in their direct pre-clinical/clinical translation [4]. The matching between the elicited *in vivo* response (e.g. angiogenic, osteo-inductive or proliferative effect) and the ECM composition could lead to the selection of a set of factors (5A) capable to induce a desired regenerative process. Ultimately, the design of the resulting customized ECM would represent a new generation of instructive materials with enhanced performance for a predictable regeneration (6). (B) The decellularization by apoptosis also offers the exciting but challenging possibility to investigate the properties of ECM from engineered/native tissues (1b), through the removal of the cellular fraction without compromising the integrity of the ECM (2b). This may allow to better understand the role and function of the different ECMs in the absence of the cellular component. The instructive capacity of such acellular constructs can be evaluated upon *in vivo* implantation in suitable models (3b) and the observed regenerative effect can either lead to a direct pre-clinical/clinical translation of the generated ECM (4), or can be matched with the respective ECM composition (5b). A combinatorial analysis of this matching may allow the identification of new signals (5b) that critically drove the regenerative process. Ultimately, these factors could be implemented within the design of customized ECM (6), converging with the approach described above (Fig. 5A) towards the generation of instructive materials that contain the necessary set of signals required for the repair of specific tissues/organs.

morphogenetic proteins), associated with cost and safety concerns [75,76], this approach relies on the embedding and presentation by the ECM of a cocktail of different factors in more physiological concentrations to activate a regenerative process (e.g., by acting on resident osteoblastic and endothelial lineage cells). In this context, human bone marrow-derived Mesenchymal Stromal Cells (hMSC) have already been used in the attempt to generate ECM with osteo-inductive properties [77,78], as a niche for reseeded MSC or for priming of endogenous progenitor cells. So far, however, the ECM deposited during *in vitro* culture could not be shown to be capable of inducing ectopic *in vivo* bone formation if deprived of the living cellular component [9]. Combining the approaches of decellularization by apoptosis with a 3D perfusion culture system [79], together with the utilization of a suitable cell source, could offer the opportunity to enhance the osteo-inductive properties of decellularized ECM. We recently reported the successful generation of an immortalized hMSC line with properties similar to the original primary cells and controlled survival. The cell line maintains the capacity to differentiate towards the osteogenic lineage, is not tumorigenic and – thanks to the implementation of a genetic device – can be pushed to programmed cell death by the delivery of a clinically approved chemical inducer [80].

Here we report that the cell line could deposit a mineralized ECM in 2D culture and still be efficiently induced towards apoptosis (>95%, Fig. 4). Moreover, when seeded on a porous ceramic scaffold within a 3D perfusion-based bioreactor system, the engineered cell line was capable to adhere, proliferate and deposit an ECM. The generated constructs could be directly and efficiently decellularized by the direct perfusion of the apoptotic-inducer through the ECM, leading to the successful generation of ECM-decorated, cell-free materials. As compared to the living counterparts, the "apoptized" tissues maintained the amount of total collagen, as representative ECM protein, in contrast to those decellularized by a conventional freeze/thaw protocol, where a marked loss of total collagen content (74%) was measured (Fig. 4). This setting thus provides a proof-of-principle for the decellularization of ECM produced by osteogenic cells following the described apoptotic *Death-engineering* strategy, in a perfusion bioreactor setup.

Further studies are required to assess (i) to which extent the apoptotic treatment may better preserve the deposited ECM, beyond total collagen, as compared to traditional protocols of decellularization and (ii) whether the resulting "apoptized" grafts have osteo-inductive properties. The paradigm could obviously be extended to other cell lines, for the deposition of a customized ECM specifically competent to induce regeneration of different tissues.

7. Conclusion & perspective

The deliberate activation of programmed cell death is here proposed as an elegant alternative to conventional approaches for decellularization of native or engineered ECM. Thanks to the possibility to specifically target the cellular component, decellularization by apoptosis-induction is expected to better preserve the integrity of the structural and instructive components of an ECM and ultimately to lead to the generation of *off-the-shelf* grafts with enhanced regenerative properties. The different apoptosis induction strategies described above offer a wide range of possibilities which could also be combined with a 3D perfusion culture system for a streamlined process implementation and/or with the use of immortalized cell lines, genetically implemented with an inducible death device.

The use of a cell line for the engineering of ECM to be decellularized would support not only repeatable production of batches with standardized properties, but also optimization and customization of the ECM regenerative potency. In fact, a cell line could be further engineered to overexpress defined factors, for example specific BMPs for enhanced osteoinduction or VEGF for more efficient vascularization. The design of customized ECM (Fig. 5A) to elicit specific repair would allow establishing a new generation of instructive materials matching the structural and molecular cues required for a predictable regeneration.

The use of apoptosis as decellularization technique goes beyond the generation of grafts with enhanced performance. The concept would also offer the unprecedented possibility to investigate the properties of decellularized but theoretically intact ECM and – by correlating an observed regenerative capacity with a specific composition – to identify a set of cues critical to elicit certain functions (Fig. 5B). The investigation and validation of the relevance of such features could then ultimately support a transition from the paradigm of decellularization of engineered biological ECM to the vision of entirely synthetic matrices, designed to contain the necessary and sufficient set of signals required for the repair of specific tissues/organs.

Author contributions

Paul Bourgine: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; Benjamin Pippenger: experimental design, collection and assembly of data, data analysis and interpretation, manuscript writing; Atanas Todorov Jr.: manuscript writing; Laurent Tchang: figures conception; Ivan Martin: conception and design, financial support, manuscript writing, and final approval of manuscript.

Acknowledgments

The research leading to these results has received funding from the European Community's Seventh Framework Programme (MultiTERM, grant agreement nr 238551). The authors acknowledge Fin-Ceramica Faenza S.p.a, Italy for the generous supply of Engipore scaffolds.

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Chapter 4

“Generation of acellular bone grafts with well-preserved properties by apoptosis-driven decellularization”

Paper under preparation

Generation of acellular bone grafts with well-preserved properties by apoptosis-driven decellularization

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ABSTRACT

The generation of tissue-engineered, decellularized bone substitutes appears as a promising alternative to existing bone repair strategies. In particular, materials combined with a cell-laid ECM have been proposed as instructive templates to guide residing cells toward bone formation. However, the instructive properties of such ECM-coated materials may highly depend on the decellularization procedure that can affect the structure and/or composition of the generated graft. The activation of programmed cell death was proposed as novel decellularization approach, in order to better preserve the ECM properties. Here, using a human death-inducible bone marrow-derived mesenchymal stromal cell (BMSC) line, we explore the possibility to generate ECM-coated constructs for bone regeneration purposes and to decellularize them by apoptosis induction. ECM-coated materials were successfully generated using a ceramic-based scaffold, by culture for 4 weeks in a 3D perfusion bioreactor. The resulting grafts were decellularized using either induction of apoptosis, or a conventional Freeze & Thaw (F&T) protocol as comparative method. While both procedures were shown to efficiently decellularize the constructs (>90% of cell death), the apoptotic method led to a better conservation of the critical ECM components (e.g. Collagens, VEGFα, IL-8, MCP-1, SDF-1 and M-CSF). The validation of this approach is expected to lead to a standardized procedure for the engineering of bone graft substitutes, which includes the combination of a standard cell source, a novel decellularization technique, a controlled 3D culture system and a clinically suitable ceramic material.

INTRODUCTION

The healing of bone remains a major clinical issue that requires the development of alternative strategies capable to promote a fast, stable and efficient repair (1). Tissue engineering approaches proposed the *in vitro* generation of bone grafts, through the seeding of osteoprogenitors -such as BMSC-, on three-dimensional (3D) scaffolds that support cell growth, differentiation and extracellular matrix (ECM) deposition (2-4). The resulting engineered grafts, containing living cells, are expected to induce tissue regeneration upon implantation into a defect site. Nevertheless, limits aroused from the lack of standardization -mainly cell source dependent (5)-, and the method only leads to an autologous exploitation of the generated bone substitute.

For these reasons, the development of decellularized grafts has received increased interests, as immune-compatible and *off-the-shelf* alternative to living grafts. The concept relies on the possibly retained osteoinductive properties of the ECM-coated scaffolding material despite the cellular depletion. The ECM is then perceived as a morphogen reservoir capable to instruct resident cells toward bone regeneration.

The success of this strategy may depend on the use of a suitable decellularization protocol, allowing the efficient removal of the immunogenic cellular materials while preserving the integrity of the deposited ECM (6), meant to drive the *in vivo* bone repair. As existing strategies were shown to vary in their efficiency and to affect the ECM structure/composition, a paradigm shift has been recently proposed by the activation of the programmed cell death as decellularization technique (7). The induction of apoptosis is expected to lead to tissue decellularization while better preserving the ECM integrity.

In this study, we aim at generating ECM-coated materials using a death-inducible BMSC line seeded and cultured on ceramic scaffolds in a 3D perfusion bioreactor. In particular, the activation of the inducible death-device was designed to serve as decellularization method. The resulting acellular grafts were compared to those obtained by F&T decellularization, in terms of cell removal efficiency and matrix preservation. We hypothesize that the apoptotic approach may better preserve the graft

integrity thus allowing for the generation of bone substitutes with an intrinsic increased regenerative potential.

MATERIALS & METHODS

Cell culture

Cell expansion

The previously generated M-SOD line (7) was cultured in complete medium consisting of α-minimum essential Medium (αMEM) with 10% fetal bovine serum, 1% HEPES (1M), 1% Sodium pyruvate (100mM) and 1% of Penicillin-Streptomycin-Glutamin (100X) solution (all from Gibco). Cells were cultured in a humidified 37C°/5% CO2 incubator and medium was changed twice in a week.

Osteogenic differentiation in 2D

M-SOD cells were seeded at 3000 cells/cm2 and differentiated for 3 weeks in osteogenic medium (OM). Osteogenic medium consisted of αMEM complete medium supplemented with 10 nM Dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate and 10 mM β-glycerophosphate (8).

Death-induction

The B/B homodimerizer (Clontech, cat# 635060) was added at 50 nM in culture medium to activate the apoptosis pathway through the dimerization of the modified caspase 9.The percentage of induced death was assessed 12 hours later by FACS analysis, after cell harvest and staining with Annexin V-APC (BD Biosciences, cat#550475) and Propidium Iodide (PI, BD Biosciences, cat# 51-66211E) in Annexin-V binding buffer (BD Biosciences, cat# 556454). Control cells were cultured in the same medium without exposure to the homodimerizer.

Generation of ECM-coated construct

Cell seeding

The 3D culture was performed using a previously developed perfusion bioreactor system (U-cup, Cellec Biotek AG), allowing controlled flow of a cell suspension or culture medium directly through the scaffold pores (9). M-SOD cells were seeded on hydroxyapatite scaffolds (Engipore®, Finceramica-Faenza, Faenza, Italy) in the form of porous cylinders of 4 mm height and 8 mm diameter. Scaffolds were seeded with $0.75*10^{6\ of}$ M-SOD cells by overnight perfusion at a superficial velocity of 1000 µm/s in proliferation medium (PR), consisting of CM supplemented with 100 nm dexamethasone, 0.1 mm ascorbic acid-2-phosphate and 5 ng/ml FGF-2. After 24 h (cell seeding phase), the superficial velocity was reduced to 100 µm/s for perfusion culture of M-SOD cells. The cell homogeneity and viability within constructs was assessed by methyl-tetrazolium staining (MTT, Sigma).

Graft generation

The protocol for the generation the of ECM-coated constructs was adapted from (10). Finally, grafts were generated by culturing M-SOD cells for 1 week in PR and 3 weeks in OM, within the 3D perfusion bioreactor system. Culture medium was changed twice a week. Samples were sacrificed at day 1, day 7 and day 28 to evaluate the cell seeding efficiency and cell proliferation.

Graft decellularization

The constructs were decellularized using the previously described apoptotic approach or a conventional F&T method. Apoptosis was induced as described above, and cell debris was removed by washing the constructs in sterile PBS in the perfusion bioreactor at 100 µm/s for 30 min at 37°C (adapted from (10)). The F&T consisted of three cycles of dry freezing in liquid nitrogen and thawing in a 37 °C water bath for 10 minutes each respectively. Samples were rinsed in double distilled water after the 1^{st} thaw in order to hypotonically lyse the cells. Subsequent to the F&T divitalization, the elimination of cellular debris was performed by a perfusion-based washing step: the constructs were placed back into the bioreactor system and perfused at 100 µm/s in PBS for 30 min at 37°C.

Ostegenic differentiation assessment

Real-time RT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA), treated with DNAse and retrotranscribed into cDNA, as previously described (11). Real-time RT-PCR was performed with the ABIPrism 77000 Sequence Detection System (Perkin Elmer/Applied Biosystem, Rotkreuz, Switzerland) and expression levels of genes of interest were normalized to GAPDH. Primers and probe sets of osteogenic genes were used as previously described (11).

Biochemical staining

Alizarin red biochemical staining was used to assess the osteogenic differentiation of the cells, as previously described (12).

Cell extraction from the 3D scaffold

Following 3D cultures, cells were extracted from the scaffold pores by sequentially perfusing a solution of 0.3% collagenase (Worthington, USA) for 40 min and 0.05% trypsin/0.53 mm EDTA (GIBCO, Switzerland) for 10 min, both at a superficial velocity of 400 µm/s. Previous studies using a similar setup indicated that the protocol allows detaching of more than 85% of the total cells in the system (13).

Osteogenic marker analysis

M-SOD cells from the 2D or 3D generated ECM were retrieved, permeabilized for 15 min in permeabilization buffer (R&D systems cat# F005) and stained at 4 °C for 30 min with an antibody against STRO-1 (R&D systems cat# MAB1038), osteocalcin (OC, R&D systems cat# IC1419P) or alkalyne-phosphatase (ALP, R&D systems cat# FAB1448A). Cells were washed and stained with an APC conjugated secondary antibody (R&D systems cat# IC002A) for 30 min and analyzed using a

FACSCalibur flow cytometer (BD Biosciences, Germany). Positive expression was defined as the level of fluorescence greater than 95% of the corresponding isotype control.

ECM characterization

DNA quantification

Constructs retrieved from the bioreactor chamber were digested with proteinase K solution (1 mg/ml proteinase K, 50 mm TRIS, 1 mm EDTA, 1 mm iodoacetamide, and 10 µg/ml pepstatin-A; Sigma–Aldrich, USA) in double distilled water or potassium phosphate buffer for 16 h at 56 °C as previously described (14). DNA quantification was performed by means of a commercially available fluorescence based kit, namely CyQUANT® Cell Proliferation Assay (Invitrogen, USA). Working solutions were prepared according to the manufacturer's protocols. The analyses were carried out measuring fluorescence with a Spectra Max Gemini XS Microplate Spectrofluorometer (Molecular Devices, USA). Excitation and emission wavelengths were respectively 485 nm and 538 nm. Samples in each plate included a calibration curve.

Collagen and calcium quantification

Total calcium was measured using a colorimetric-based assay. Briefly, calcium present in the mineralized extracellular matrix was solubilized in 0.5 N hydrochloric acid for 4h at 4°C before quantification (Total Calcium Assay, Randox, UK). Total collagen content was determined using the Sircol assay (Biocolor, UK) after samples solubilization in 3% v/v acetic acid (Fluka, Switzerland) and 0.01% w/v pepsin (Sigma–Aldrich, USA) overnight at 4 °C. The calcium/collagen concentration was assessed using a Spectra Max 190 microplate colorimeter (Molecular Devices, USA) following the parameters provided in the respective assay kits.

Scanning electron microscopy (SEM)

For SEM, cell-seeded constructs were fixed overnight at 4 °C with 4% formaldehyde and washed with PBS. Samples were gradually dehydrated with 30-50-70-90-100% ethanol, coated with gold and imaged with a Philips XL 30 ESEM microscope.

Affymetrix-protein quantification

Prior to and after decellularization procedures, the generated ECM coated grafts were analyzed for their content of a panel of growth factors, chemokines, and metalloproteinases, according to the manufacturer's instructions (Procarta Immunoassay Kit; Panomics).

Statistical analysis

Data are presented as means ± standard deviation. The significance of differences was evaluated using Mann Whitney tests, with $p< 0.05$ considered to indicate statistical significance (GraphPad Prism 5).

RESULTS

Osteogenic differentiation of M-SOD cells and generation of 2D decellularizable ECM

M-SOD cells were cultured for 3 weeks in osteogenic medium to assess their differentiation capacity. Upon 2D culture, cells displayed a typical kinetic of osteogenic differentiation (figure 1A) marked by a decrease in the percentage of cells expressing the stro-1 progenitor marker (9.6-fold) and ALP protein (5.3-fold) and increase of the later marker OC (9.9-fold) from 2 to 3 weeks of culture. The differentiation was confirmed by the upregulation of key osteogenic genes (figure 1B) assessed after 3 weeks of osteogenic differentiation (6.1, 10.9 and 8.1 fold higher levels than prior to induction for ALP, OC and BMP-2 respectively).

During 2D culture, M-SOD cells were shown to deposit a collagen-rich ECM (figure 2B), that mineralized in the presence of osteogenic medium (figure 1A) as confirmed by the high calcium

content (52.6 µg/mL of culture, figure 2C). Importantly, the ECM secreted by the engineered M-SOD cells could be decellularized by activation of the inducible-death system integrated within those cells. The apoptosis induction was shown to result in an efficient cell-killing (94.9%, figure 2D), thus demonstrating the possibility to generate decellularizable ECM in 2D.

Engineering of ECM-coated constructs

Cells were seeded on ceramic scaffolds and cultured for 4 weeks in 3D perfusion-based bioreactors in order to generate grafts decorated with a mineralized ECM (figure 3A). A proliferative medium (PR) was initially supplemented for 1 week to support cell growth and scaffold colonization. The medium was then switched to an osteogenic medium (OM) for 3 weeks to induce cell differentiation and matrix deposition.

Following dynamic seeding on ceramic scaffold, cells continuously proliferated with the largest increase in cell number observed during the 1 week of culture when supplemented with proliferative medium (0.48 PD/day, figure 3C). During the 3 weeks of osteogenic differentiation, cells continued to proliferate though they displayed a reduced growth rate (0.19 PD/day, figure 3C).

After 4 weeks of 3D culture, the constructs contained more than 6 million cells (figure 3C) rather homogeneously distributed within the scaffold pores (figure 4A). M-SOD cells were efficiently differentiated as assessed by the pattern of expression of ALP, stro-1 and OC protein (figure 4B). The gene expression analysis confirmed the successful differentiation of M-SOD in the 3D scaffolds as a strong upregulation of key genes involved in the osteoblastic differentiation was observed. As compared to expanded M-SOD, cells retrieved from the generated constructs upregulated ALP, OC, BSP, Cbfa-1, and OSX by a 12.1-, 3.2-, 6.1-, 2.1-, and 17- fold respectively (figure 4C). Interestingly, a strong MMP-13 over-expression was also observed (17.1- fold, figure 4C), which is required for collagen matrix remodeling toward bone mineralization (15).

Taken together, these data demonstrate the successful differentiation of M-SOD during the 3D perfusion culture on ceramic scaffolds.

Decellularization of the generated grafts

After 4 weeks of 3D perfusion culture, the grafts consisted in a thick ECM coating the scaffolding material (supp. data 1) but still containing the living cellular fraction. In order to remove the cell compartment, constructs were decellularized either by repeated freeze and thaw (F&T) cycles or by apoptotic induction through the supplementation of the chemical inducer of dimerization (CID) activating the M-SOD suicide device.

Both decellularization processes led to an efficient cell death as qualitatively assessed by MTT staining of the construct (figure 5A). Importantly, treatments led to a similar cell killing efficiency (92.3% and 98.4% for F&T and CID respectively, figure 5B) while only 14% of apoptotic cells were measured in non-decellularized constructs. In particular, the perfusion system allowed for an efficient removal of the cellular fraction following F&T or CID treatment with respectively 81.4% and 72.5% of DNA removal (figure 5C). Thus, both the apoptotic induction and F&T method allowed for a comparable and efficient decellularization of the generated grafts.

Impact of the decellularization treatments on the produced ECM

Although both decellularization methods led to a successful cell removal, the success of a decellularization strategy also relies on its ability to preserve the ECM structure and composition.

After 4 weeks of culture, cells deposited a collagen-rich ECM (21.7µg/construct, figure 6A) coating the ceramic material (supp. data 1). However, the F&T decellularization resulted in an important loss of collagen content (74% of the pre-decellularization content) with only 5.6µg/construct remaining after treatment. Instead, the apoptotic induction process seemed not to affect the structural composition of the ECM, as no loss of collagen was observed (21.6µg/construct, figure 6A).

The F&T treatment also led to an important loss of key ECM proteins (figure 6B). A strong reduction of interleukin 8 (IL-8, 17.4% of the pre-decellularization content), monocyte chemotactic protein-1 (MCP-1, 14.2%), osteoprotegerin (OPG, 13.1%), vascular endothelial growth factor α (VEGFα, 34%), stromal cell-derived factor 1 (SDF-1, 13.8%), and macrophage colony-stimulating factor (M-CSF, 79.4%) content was observed. The apoptotic method was shown to better maintain the ECM

proteins as no reduction of the IL-8, M-CSF and VEGFα content could be observed. A marked decrease of MCP-1 (66.3%), OPG (25.9%) and SDF-1 (48%) was observed, but to a lower extent as compared to the F&T treatment. In particular, the decrease of OPG, inhibitor of osteoclastogenesis (31), could be in part explained by the possible ligation of this soluble receptor to its cell-bound receptor (32). Thus the removal of the cellular fraction from the graft may also remove the bound OPG.

DISCUSSION

Using a previously described death-inducible BMSC line (7), we investigated the possibility to generate ECM-coated material and subsequently decellularize them by apoptosis induction.

In this experimental setup, the M-SOD line was successfully differentiated toward the osteoblastic lineage and was shown to be able to deposit a collagen-rich, mineralized matrix. Importantly, the cells could be efficiently induced toward death after ECM deposition, thus validating for the first time the deliberate apoptosis activation to decellularize a pre-engineered tissue. Importantly, the proposed approach was shown to induce a high cell killing efficiency and allowed for an efficient removal of the cellular fraction. In fact, the decellularization efficiency was comparable to the well-established F&T technique (10). However, the decellularization by apoptosis was shown to better preserve the deposited ECM. In particular, collagens –the most important ECM structural proteins (20), were shown to be conserved upon decellularization treatment, thus supporting a structural preservation of the graft.

The apoptotic method also led to the better conservation of key ECM-embedded cytokines and morphogens, toward the use of the graft as instructive template. Indeed, crucial steps to drive a bone healing process include an inflammation phase and the vasculature recruitment (21-23). The apoptotic method led to the better preservation of IL-8 and MCP-1, both mediators of immune reaction (24, 25), as well as involved in the recruitment of host cells (26). Moreover, apoptosis preserved the VEGFα and SDF-1 contents which may be crucial for the successful engraftment of the constructs, as strong inducers of angiogenesis (27, 28). SDF-1 was also shown to drive the migration

of hematopoietic cells (29) which can be of importance toward the reconstitution of the bone marrow niche. In addition, M-CSF is involved in HSC differentiation (30). Overall, the superiority of the apoptotic method supports its use for the generation of osteo-inductive grafts.

Using a perfusion bioreactor system, M-SOD cells were shown to actively proliferate in 3D culture after seeding on a ceramic scaffold, leading to the generation of homogenously ECM-coated constructs. If a direct comparison of 2D and 3D culture conditions might not be relevant, a superior differentiation of M-SOD cells was clearly observed in the 3D setup (29.9% of OC expression in 2D versus 82.3% in 3D). This could be partially explained by the flow-induced mechanic stimulation offered by the perfusion device (16) together with the enhancement of mass transport.

The ceramic scaffold was selected for this study with the rational to provide the essential calcium-phosphate substrates required for new bone formation (18, 19). Moreover, ceramic scaffolds are generally highly biocompatible and therefore promising toward the direct clinical translation of the approach (18). Obviously, the validation of the M-SOD cells in combination with other scaffolding material may be of high interest to possibly extend the range of applications.

The instructive potential of the here engineered constructs still needs to be determined by assessing a possibly faster and/or increased bone regeneration than non-ECM coated or F&T treated constructs. Nevertheless, this approach displays key advantages over pre-existing bone repair strategies. Mainly, fully engineered delivery systems only allow for the release of a single or limited combination of growth factors, and the associated concentration and kinetic of delivery is subject to tuning difficulties. Instead, a natural ECM can be perceived as a growth factor reservoir that contains a complete set of morphogens/cytokines that can be delivered and presented at physiological –thus safe– dose. On the other hand, fully natural ECMs derived from decellularized native tissues (e.g. bone) are of limited availability and have lost in part their regenerative potential during the decellularization process. Thus, the proposed approach consisting in the use of cell line to decorate a synthetic scaffold with a natural ECM may solve availability, safety and quality issues for the effective clinical use of the resulting material.

CONCLUSION

The use of the M-SOD line offers both an unlimited and standard cell-source while intrinsically representing an innovative decellularization tool. Together with a 3D-perfusion system that allowed enhancement of performance while increasing the convection of the apoptotic inducer and removal of cell debris, the strategy represents a significant advance for the development of an automated streamlined procedure for the standard generation of bone-graft substitute. Ultimately, the concept could be applied for the generation and subsequent decellularization of other types of tissues.

ACKNOWLEDGEMENTS

This work was supported by the European Community's Seventh Framework Program (MultiTERM, grant agreement number 238551).

DISCLOSURE OF POTENTIAL CONFLICTS OF INTERESTS

The authors declare no potential conflicts of interest.

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FIGURES LEGENDS

Figure 1: Osteogenic differentiation of M-SOD in 2D culture. M-SOD cells were successfully differentiated as demonstrated by the strong expression of the early ALP protein at 2 weeks (46.6%, A) and strong of the later OC marker (29.9%, A). The progenitor marker stro-1 decreases upon differentiation. The over-expression of key osteogenic genes measured after 3 weeks (B) confirmed the osteogenic differentiation of M-SOD cells (6.1, 10.9 and 8.1 fold difference for ALP, OC and BMP-2 respectively). *, $p<0.05$.

Figure 2: Generation of decellularizable osteogenic matrix in 2D. Upon 3 weeks of osteogenic differentiation, M-SOD cells were shown to be capable to deposit a mineralized, collagen-rich matrix as assessed by total collagen (A), alizarin-red staining (B), and calcium deposition (C). The secreted ECM could also be efficiently decellularized by apoptosis induction (94.9%, C). Size bar = 1cm

Figure 3. 3D culture of M-SOD in perfusion bioreactor. Experimental set up leading to the generation of ECM-coated scaffold followed by their subsequent decellularization using a Freeze& Thaw (F&T) or apoptotic approach (A). The cell line demonstrated a continuous proliferation, reaching more than 6 million cells after the 4 weeks of culture (B).

Figure 4: Differentiation of M-SOD in 3D perfusion bioreactor. After 4 weeks of culture, scaffolds were homogenously populated with living cells (MTT staining, A). The strong OC (82.3%) and ALP (26.6%) expression and assessed of the successful cell differentiation, with resident progenitor cells revealed by low Stro-1 expression (3.7%, B). The gene expression levels of key osteogenic genes (C) confirmed the osteogenic differentiation of M-SOD cells in bioreactors (12.1-, 3.2-, 6.1-, 2.1-, 17-, 17.1- fold difference for ALP, OC, BSP, Cbfa-1, OSX and MMP-13 respectively). Size bar = 0.2cm. *, $p<0.05$.

Figure 5: Decellularization of ECM-coated scaffolds. Both decellularization protocols led to an efficient cell killing, as assessed by MTT staining (A). Quantification by flow cytometry (B) revealed a similar percentage of death-induction in Freeze & Thaw (F&T, 92.3%) and apoptosis-induced samples (+CID, 98.4%). The decellularization processes also led to an efficient removal of the DNA material (C, 81.4% and 72.5% respectively) with only 3309.5 ng and 4883.8 ng after F&T and apoptotic treatment respectively. Size bar = 0.2cm

Figure 6: Effect of decellularization treatments on the ECM. The decellularization by F&T led to a severe loss of collagen (74%, A) while the apoptotic treatment did not impair the content (A). The quantification by Luminex analysis confirmed the better preservation of ECM proteins using the apoptosis treatment. *, $p<0.05$. $n\geq4$.

Supplementary data 1: Scanning electron microscopy of empty (A) and 3D engineered construct (B). Engineered constructs were seeded with M-SOD cells and cultured for 4 weeks in perfusion bioreactor, leading to the coating of the material with ECM.

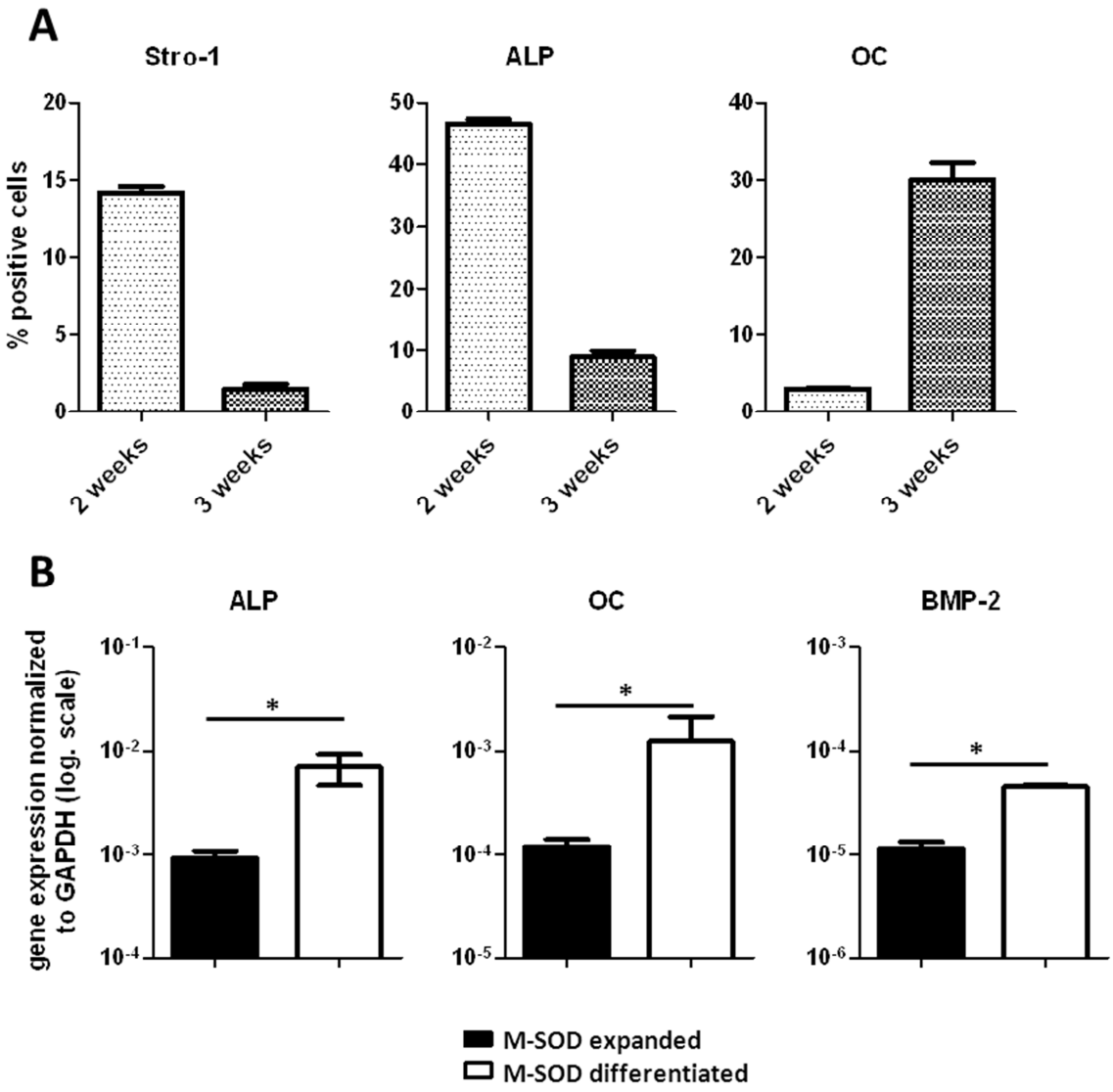

A
Stro-1
ALP
OC
% positive cells
20
15
10
5
0
50
40
30
20
10
0
40
30
20
10
0
2 weeks
3 weeks
2 weeks
3 weeks
2 weeks
3 weeks
B
ALP
OC
BMP-2
gene expression normalized to GAPDH (log. scale)
10-1
10-2
10-3
10-4
10-2
10-3
10-4
10-5
10-3
10-4
10-5
10-6
*
*
*
M-SOD expanded
M-SOD differentiated

Figure 1

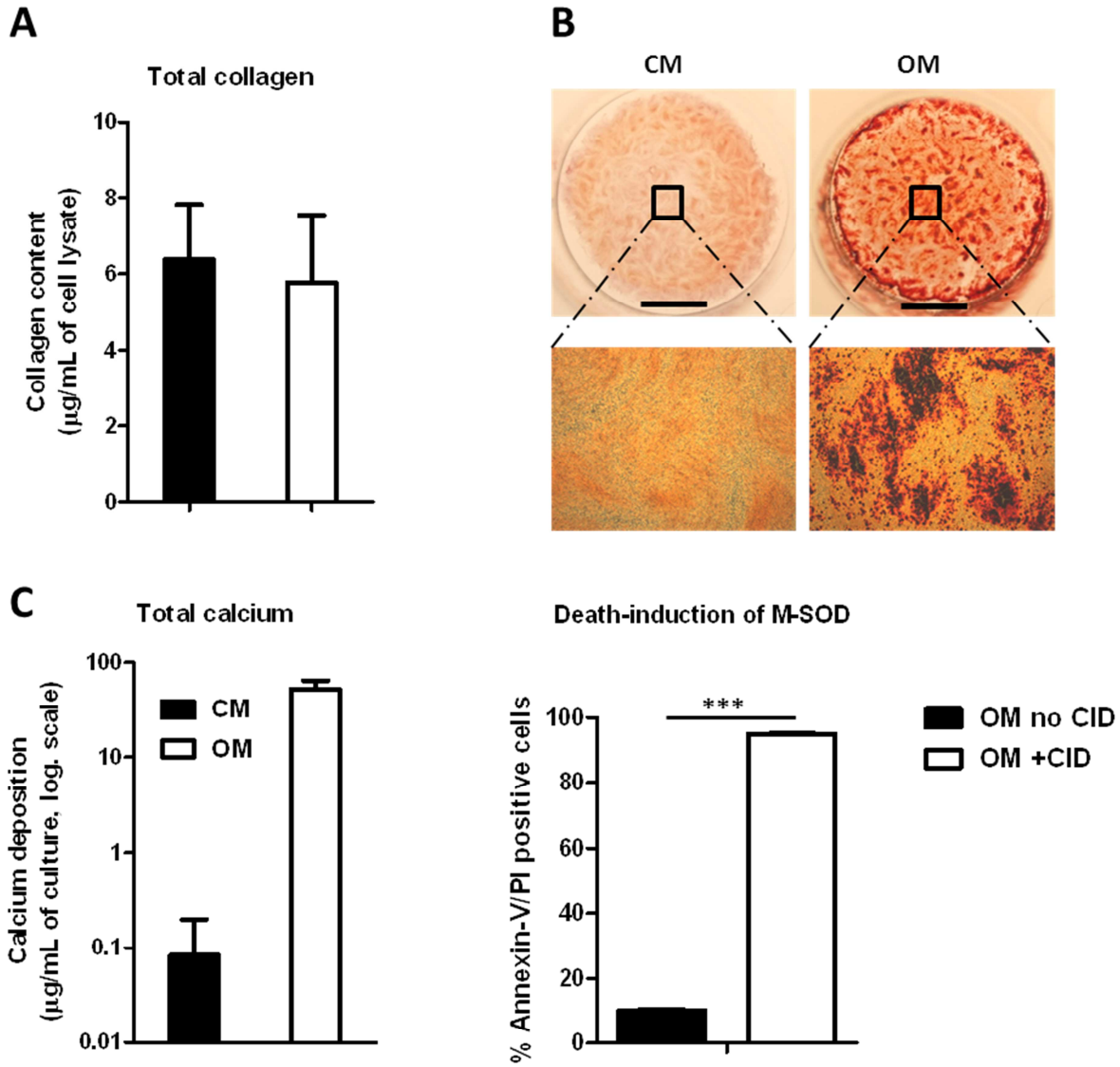
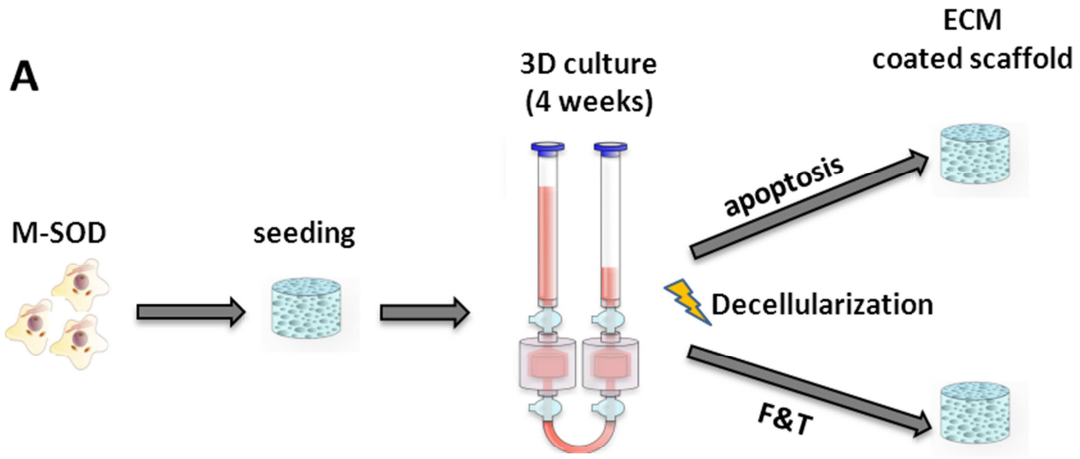

A
Total collagen
Collagen content (µg/mL of cell lysate)
10
8
6
4
2
0
B
CM
OM
C
Total calcium
Calcium deposition (µg/mL of culture, log. scale)
100
10
1
0.1
0.01
CM
OM
Death-induction of M-SOD
% Annexin-V/PI positive cells
100
80
60
40
20
0
***
OM no CID
OM +CID

Figure 2


A
M-SOD
seeding
3D culture
(4 weeks)
ECM
coated scaffold
apoptosis
Decellularization
F&T

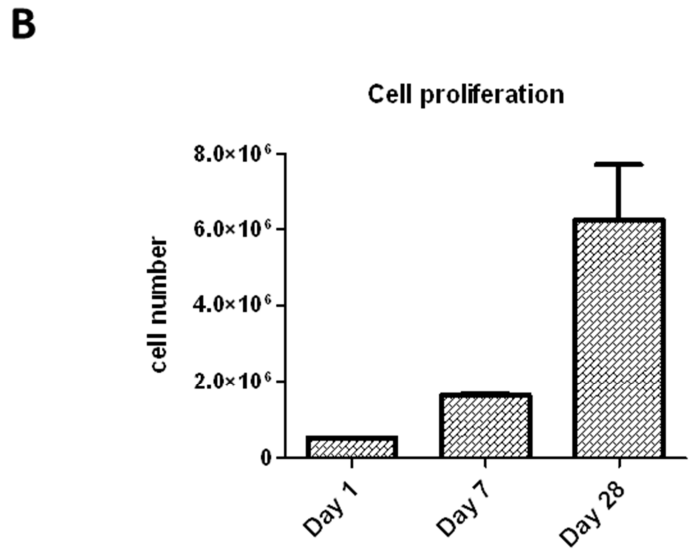

B
Cell proliferation
cell number
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
Day 1
Day 7
Day 28

Figure 3

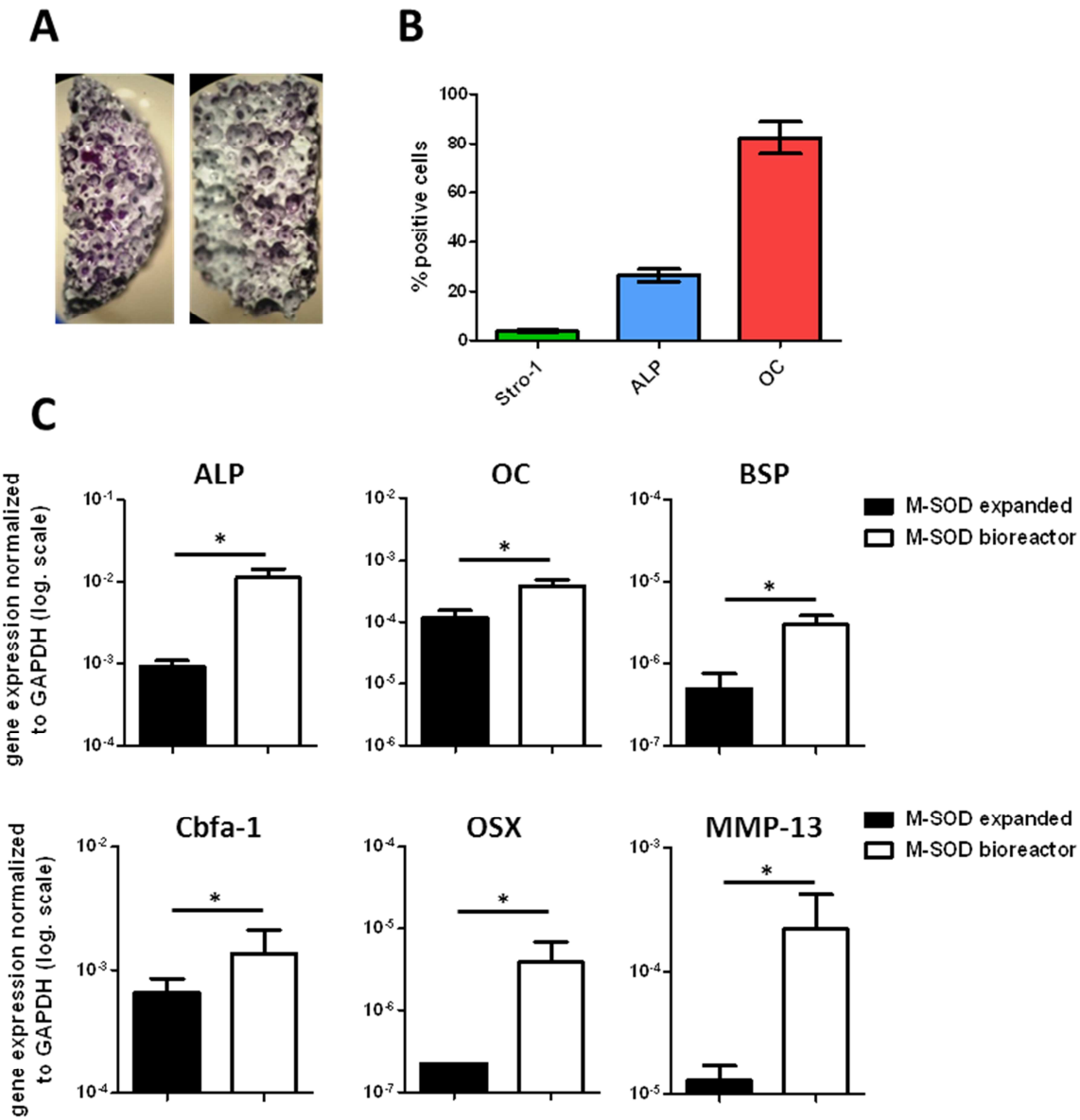

A
B
% positive cells
100
80
60
40
20
0
Stro-1
ALP
OC
C
ALP
OC
BSP
gene expression normalized to GAPDH (log. scale)
M-SOD expanded
M-SOD bioreactor
Cbfa-1
OSX
MMP-13
gene expression normalized to GAPDH (log. scale)
M-SOD expanded
M-SOD bioreactor

Figure 4

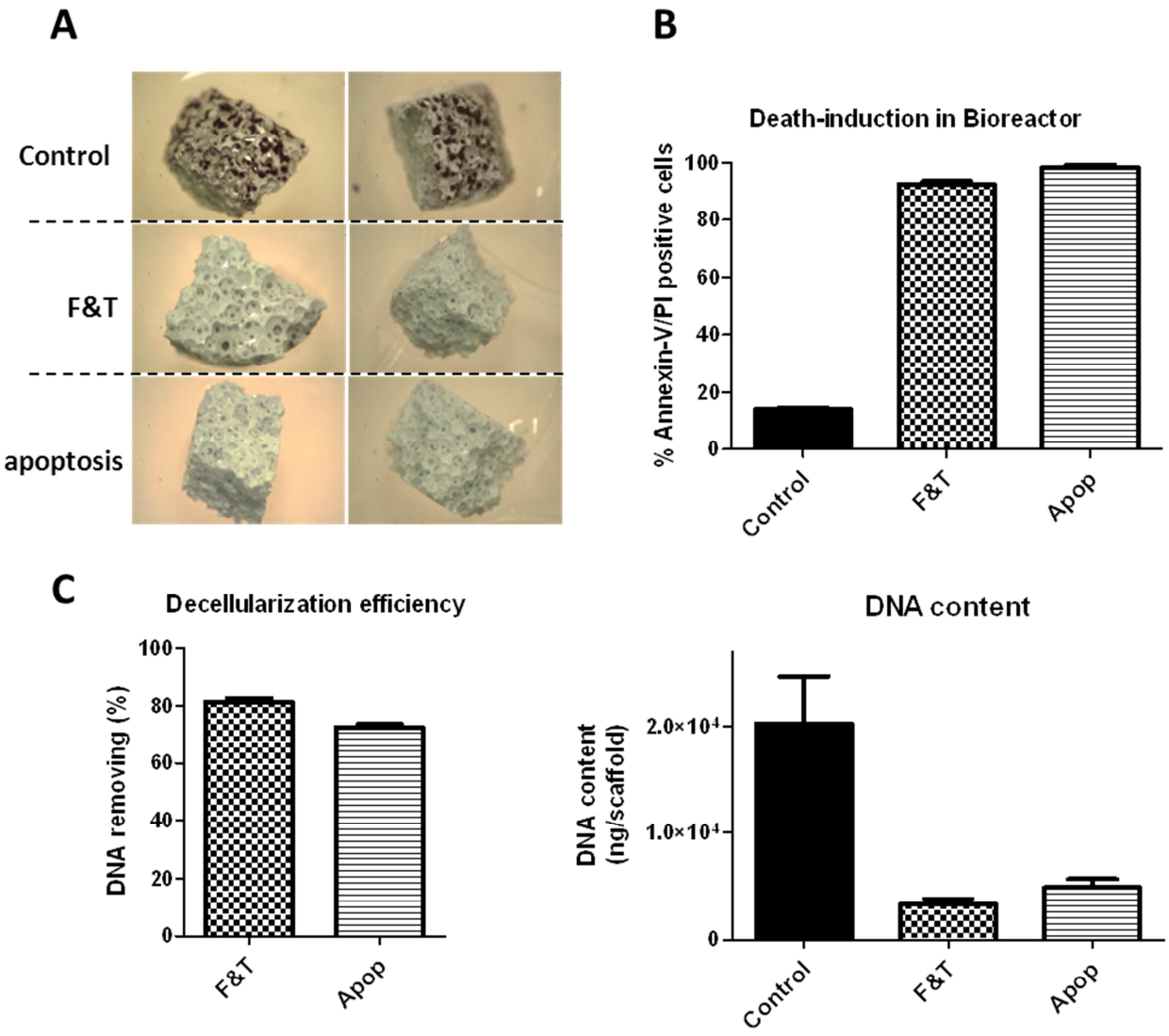

A
Control
F&T
apoptosis
B
Death-induction in Bioreactor
% Annexin-V/PI positive cells
100
80
60
40
20
0
Control
F&T
Apop
C
Decellularization efficiency
DNA removing (%)
100
80
60
40
20
0
F&T
Apop
DNA content
DNA content (ng/scaffold)
2.0×10⁴
1.0×10⁴
0
Control
F&T
Apop

Figure 5

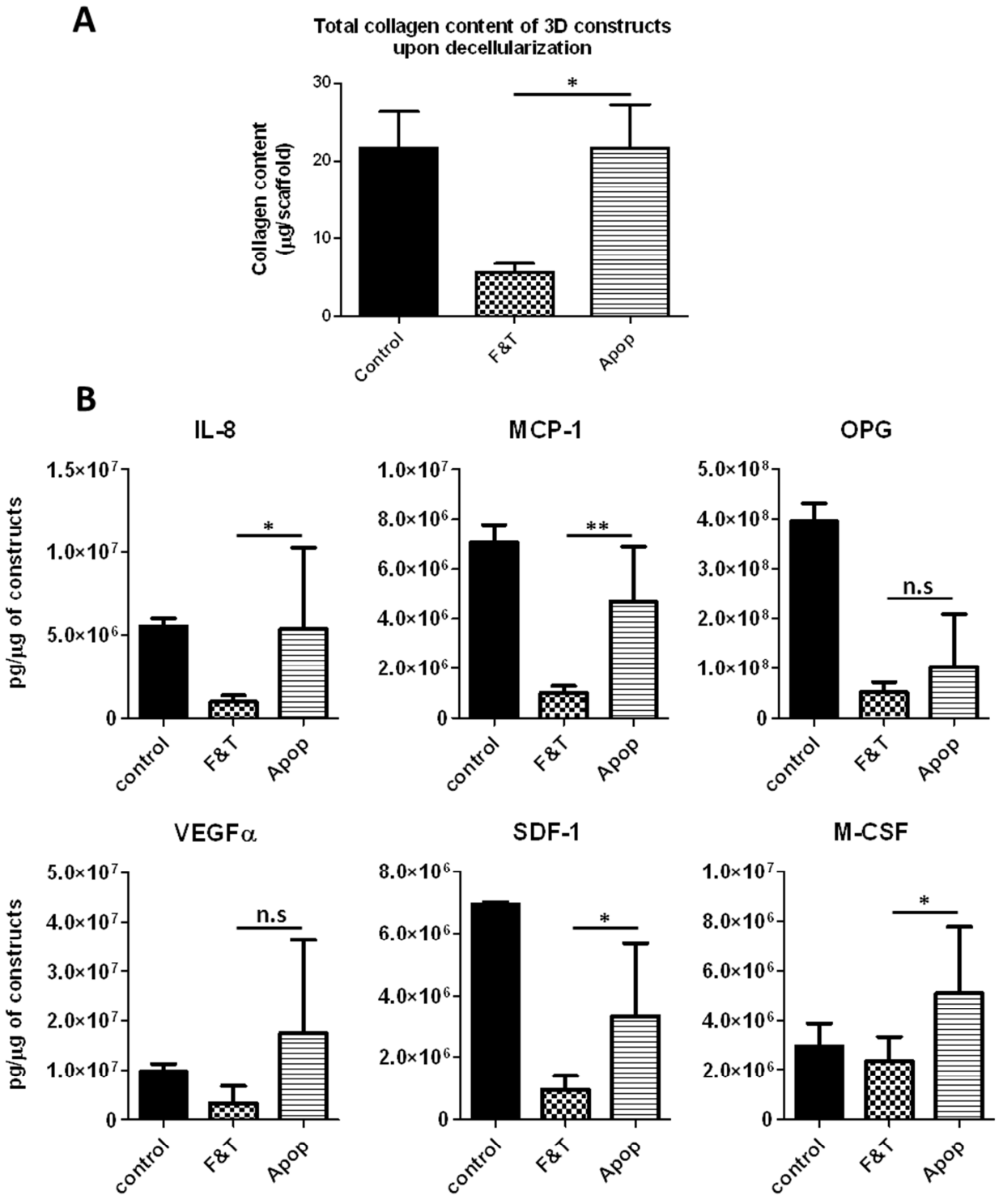
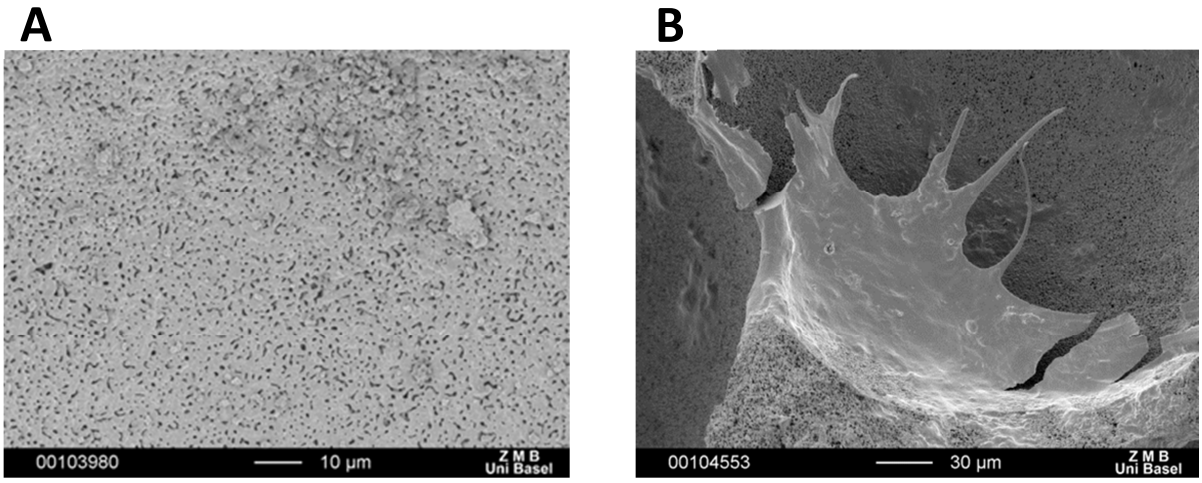

A
Total collagen content of 3D constructs upon decellularization
Collagen content (µg/scaffold)
30
20
10
0
*
Control
F&T
Apop
B
IL-8
MCP-1
OPG
pg/µg of constructs
1.5×10^7
1.0×10^7
5.0×10^6
0
*
control
F&T
Apop
1.0×10^7
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
0
**
5.0×10^8
4.0×10^8
3.0×10^8
2.0×10^8
1.0×10^8
n.s
VEGFα
SDF-1
M-CSF
5.0×10^7
4.0×10^7
3.0×10^7
2.0×10^7
1.0×10^7
n.s
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
*
1.0×10^7
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
*

Figure 6



Supplementary data 1

Chapter 5

Conclusion & Perspectives

Impact on bone tissue engineering…

The need for bone graft substitutes is in constant increase, driven by the gain in life expectancy that results in the aging of the population. As existing repair strategies were shown to be associated with subsequent drawbacks, the healing of bone remains a major and challenging health problem that requires the development of more effective regeneration therapies.

Progress in stem cell biology and material science led to the emergence of different tissue engineered-based approaches, holding great promise. However, only few of them led to clinical translation as clear limitations still need to be overcome, including the demonstration of a higher regenerative potential over conventional treatment, the cost-effectiveness, the standardization and the possible immunogenicity in a non-autologous setting.

This thesis aimed at incorporating these pre-identified requirements within the elaboration process of an ECM-coated ceramic material, to be used as bone graft substitute. In fact, this strategy presents the advantage to directly address immunogenicity issues as avoiding the previously necessary patient-graft matching, toward the production of a universal graft.

In particular, chapter 1 addresses standardization issue arising from the use of conventional cell sources, through the generation of a well-characterized BMSC cell line. The contribution to the state-of-the-art also includes the possible control of the cell survival through the inducible death-device implementation, thus overcoming typical concerns associated with the use of immortalized cells.

As the performance of a graft is directly linked to its composition, we aimed at improving the decellularization treatment that undoubtedly affects the integrity of a graft, thus addressing quality requirements. Chapter 2 describes an innovative concept consisting in the activation of the natural programed cell death pathway to decellularize an engineered tissue. This original approach was proposed in association with a perfusion bioreactor system in order to efficiently remove the cellular fraction while better preserving the composition and possibly associated regenerative potential of the implant.

Finally, chapter 3 demonstrated the successful combination of the developed cell line and apoptotic approach for the generation and subsequent apoptosis-driven decellularization of engineered

construct, within a 3D perfusion bioreactor. This study validates the superiority of the apoptotic approach over existing protocols in terms of ECM preservation. In particular, the developed approach presents key advantages as the acellular graft is generated and subsequently decellularized within a single, closed culture system. As a consequence, manipulation steps are limited, which is of high interest toward the development of an automated, streamlined process for bone grafts production. Hence, this development process combined with the use of a cell line represents an important step toward a cost-effective graft production.

Overall, the developed strategy allowed bypassing immunogenicity issues, standardization and cost-effectiveness issues by relying on both a novel cell source and a new decellularization protocol. As a final validation, the regenerative potential of ECM-coated constructs has to be evaluated in order to clearly demonstrate the superior potential of these implants. This is currently being performed using a critical sized cranial defect model, in nude rat animal. This may lead to the development of an alternative bone repair treatment as compared to current strategies based on drug-delivery systems, the use of autografts/decellularized native bone and BMPs delivery.

…beyond the bone

Through the generation and validation of innovative tools, this work offers a novel protocol for tissue-engineering purposes. Hence, the described method -here explored in the context of bone tissue engineering- could be extended to the engineering of other types of tissues, such as cartilage, skin or tendon.

Importantly, the cell-survival control allows bypassing limitations typically arising from the use of genetically modified cells, often associated with risks of tumorigenicity. On one hand, the immortalization allows not being time-restricted in the manipulation and selection of transformed cells, as no alterations of cell's behavior/capacity can result from the population aging. On the other hand, the death-device allows not being limited in the number of genetic manipulation as far as cell's survival remains under control with no signs of malignancy.

This opens large perspectives, like the full engineering of a cell line by incorporating a panel of new functions, from the controlled release of suitable factors to the implementation of complex tracking systems. Cells can then be perceived as a biological tool, which can be fine-tuned in order to behave as a personally designed and fully predictable entity. In this regards, this strategy displays important similarities with current synthetic/systemic biology approaches. Together with the constant evolution of molecular biology techniques, targeting the precise insertion of genetic devices at specific loci site to avoid both key regulatory genes breakdown and transgenes silencing, this paves the road to the generation of stable and safe cellular tools for regenerative medicine.

As an applied consequence, the re-engineering of cells could be performed in order to drive the synthesis of decellularizable, customized (enriched or depleted) ECMs. In fact, going even beyond the generation of grafts for a custom regenerative performance, the concept could also offer the unprecedented possibility to investigate the role of the ECM and its molecular components in tissue homeostasis. By preserving its integrity, ECM-enriched in specific factors but depleted of the living compartment could be evaluated by correlating an observed regenerative capacity with a specific ECM composition, as described in chapter 2. Eliminating the cellular player may allow better dissecting the role of the ECM and associated signals involved in regeneration or other instructive/maintenance processes.

To conclude, the depicted thesis can be perceived as the development of a tools & protocol set exploited for bone repair, but also proposed as a working platform for basic research investigations.

Curriculum vitae

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Thesis title: “Study of Antigen Processing in Antigen Presenting Cells of healthy donors and HIV-1 positive patients”

2007 ► 2008
iGEM competition organized at the Massachusetts Institute of Technology, Boston, USA.

Project title: “Conception of a cell division counter in the yeast model”

Language

Excellent command of both spoken and written English
German speaking / writing notions
French as native language

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Animation diploma (BAFA)
Musical diploma
Phlebotomist certificate

Publication List

Combination of immortalization and inducible death strategies to generate a human mesenchymal stromal cell line with controlled survival
Paul Bourgine, Clementine Le Magnen, Jeroen Geurts, Arnaud Scherberich, Ivan Martin. Stem Cell Research, submitted.

Tissue decellularization by activation of programmed cell death
Bourgine PE, Pippenger BE, Todorov A Jr, Tchang L, Martin I.
Biomaterials. 2013 Aug;34(26):6099-108.

Engineering of a functional bone organ through endochondral ossification
Scotti C, Piccinini E, Takizawa H, Todorov A, **Bourgine P**, Papadimitropoulos A, Barbero A, Manz MG, Martin I. Proc Natl Acad Sci U S A. 2013 Mar 5;110(10):3997-4002.

Selected Abstracts and Conferences Attended

Generation and characterization of a bone marrow derived mesenchymal stem cell line, comparison to the primary counterpart. **P. Bourgine**, S. Güven, A. Scherberich, I.Martin.
Swiss Stem Cell Network, Lausanne-Switzerland, 2011.

Generation and characterization of immortalized bone marrow-derived mesenchymal stem cells, comparison to their primary counterparts. **P. Bourgine**, S. Güven, A. Scherberich, I. Martin.
United Kingdom National Stem Cell Network, York-England, 2011.

Short, synthetic collagen type I peptides: an alternative to bone morphogenetic proteins to stimulate the osteoblastic differentiation of human Mesenchymal Stromal Cells? **P. Bourgine**, A. Scherberich, S. Feliciano, I. Dunkley, T. Smith, A. Papadimitropoulos, I.Martin.
Swiss Stem Cell Network, Zürich-Switzerland, 2011.

Engineering of a human death-inducible Mesenchymal Stem Cell line: towards standardized and safe cellular therapy. **P. Bourgine**, V. Mele, E. Trella, A. Scherberich, I. Martin.
International Society for Stem Cell Research, Yokohama-Japan, 2012

Engineering of customizable devitalized extracellular matrices using clonal, death-inducible, immortalized human mesenchymal stromal cells. **P. Bourgine**, B. Pippenger , S. Feliciano, S. Güven, A. Scherberich, I. Martin.
Tissue Engineering and Regenerative Medicine International Society, Vienna-Austria, 2012.

Devitalization by apoptosis-induction for the generation of extracellular matrices with well-preserved instructive properties. **P. Bourgine**, B. Pippenger, A. Scherberich, I. Martin.
Tissue Engineering and Regenerative Medicine International Society, Istanbul-Turkey, 2013.

Patent application

Immortalized Mesenchymal Stem Cells- PCT/EP2013/062162. **P. Bourgine**.