# 6 General Discussion

## 6.1 OVERVIEW

Bone fracture healing and bone-related disorders such as osteoporosis or bone metastasis are major health issues. Bone tissue engineering emerged in the last decades as promising therapeutic strategy by combining mesenchymal stromal cells, scaffolds and extracellular matrix to reproduce native tissue microenvironment and favor bone regeneration [1]. Understanding the molecular mechanisms underlying how the extracellular environment influences stem cell behavior is crucial to ameliorate the successful application of bone tissue engineering.

Throughout this thesis, we investigated how the extracellular environment actively regulates mesenchymal stromal cell behavior. In **Chapter 2** and **3** we introduced three *in vitro* models of devitalized osteoblast-derived ECMs to study how they direct the osteogenic differentiation of MSCs. The protein composition of the ECMs was extensively investigated by mass spectrometry to identify regulatory candidates that modulate matrix mineralization. In **Chapter 4** we investigated how the osteogenic differentiation and matrix mineralization were influenced by a 2-day treatment of Activin-A, by studying influences on osteoblast gene expression and miRNA profile by microarray analysis. In **Chapter 5** we studied how the kinome profile of MSCs is affected by the adhesion to the osteoblast-derived ECM in comparison to a titanium surface, by using PamChip kinase array technology. Here, in Chapter 6, we analyze strengths, limitations and challenges of our studies, and focusing on future applications and perspectives.

## 6.2 CELL-DERIVED ECMs: THERAPEUTIC POTENTIAL AND CHALLENGES

Decellularized ECMs can be key players in tissue engineering due to their cell-instructive role [2, 3]. In the last decades, cell-derived ECMs have been proposed to direct stem cell function as alternative for native tissue-derived ECMs (reviewed on [4, 5]. With this purpose, in **Chapter 2** we presented an osteoblast-derived ECM, that we obtained by culturing MSCs with osteogenic inducers, and before the onset of mineralization, we devitalized the cells by freeze/thaw cycles, followed by DNAse treatment and extensive washings with PBS, aiming to remove most of cell debris while leaving the ECM structure intact [6-8]. This combination of treatments was previously successfully used, though with different set up [9, 10]. Alternatively, $NH_4OH$ followed by Triton-X and PBS washings have also been employed [11-15], deoxycholate [16], or cold EDTA to remove cellular components [17]. A combination of physical (freeze/thaw cycles) and chemical treatments was used in most cases to produce cell-derived ECMs. We showed that cell-derived ECM enhanced the os-

teogenic potential of MSCs, which is in line with previous studies [9, 10, 18-21] and highlighting the fact that similar results were obtained despite different devitalization treatments and different species that were employed [22]. Freeze/thaw cycles as devitalization treatment was previously shown to damage the fibrillary structures of the ECM [23]. Despite this, in **Chapter 2** we showed that the ECM exhibited a rough and fibrillar surface by AFM and SEM and had a rich protein composition by mass spectrometry, being crucial to direct cell behavior, thus confirming freeze-thaw as efficient and cost-effective devitalization treatment. We focused only on the ECM composition after the devitalization procedure, as substrate for MSC cultures. However, a comparison of the ECM compositions obtained by different devitalization treatments would be worth being investigated, to study the potential enrichment of candidate proteins.

In **Chapter 2** we showed that the osteoblast-derived ECM increased the percentage of proliferating MSCs already after 1 day of culture. Therefore, the devitalized ECM could be used as alternative substrate for stem cell *ex-vivo* expansion, as already hypothesized [4]. This is supported by other studies, which showed that MSC stemness was maintained when MSCs were expanded on ECMs rather than on standard culture conditions [11-13]. Moreover, cell-derived ECMs have been shown to support MSC and HSC expansion, by reproducing the physiological stem cell niche [21] and to rescue cellular senescence, by preventing loss of differentiation potential during *ex-vivo* expansion and rejuvenating aged MSCs [13-15]. We detected antioxidant proteins such as SOD1 in the ECM but also in the human bone proteome by mass spectrometry [24]. Low oxygen tension was previously shown to inhibit osteoblast differentiation and matrix mineralization, by altering ECM composition [25, 26]. For this reason, studying the impact of hypoxia during ECM deposition, but especially the effect of the ECM deposited in lower oxygen tension on osteogenic differentiation of fresh MSCs, would be of great interest to tackle aging issues.

Cell-derived ECMs have been proposed for basic research to study the role of ECM during the steps of tissue development, or to investigate specific region of organs, such as stem cell niches, being more suitable than native decellularized organs [4, 9, 27]. Furthermore, they can be used to study disease progression as ECM is altered in pathological states such as fibrosis or osteoarthritis [5, 28]. For instance, many ribosomal proteins were detected in nonmin-ECM in **Chapter 3**. Alterations of bone marrow-stroma are crucial for pathogenesis of ribosomopathies [29]. Thus, we can envisage that patient derived-ECMs could be used to study the progression of the pathology. Moreover, decellularized ECM can be patient-specific [30] and be derived from different cell types and tumor cells [31], opening up avenues for a broad range of applications.

In conclusion, we and others have shown the many applications for cell-derived ECMs. Despite having lower mechanical properties than native decellularized organs, they can be used as coatings to ameliorate synthetic scaffold in tissue engineering, towards ECM-mimetic strategies to favor tissue regeneration [5, 19, 23]. Further research is still needed to find means to generate large quantities of ECM, though strategies such as culture of cell lines transduced with telomerase reverse transcriptase (hTERT) have been proposed [22].

## 6.3 EXTRACELLULAR MATRIX PROTEOME

Cell-derived ECMs are readily available and highly customizable [5]. In **Chapter 3** we modified the composition of cell-derived ECM presented in Chapter 2, by altering culture conditions of ECM-secreting MSCs. We cultured MSCs without osteogenic inducers to create a nonmin-ECM and an Activin-A ECM by treating MSCs with osteogenic inducers in presence of Activin-A. All three ECMs were able to induce a higher matrix mineralization by MSCs than standard culture conditions (without ECM), but the ECM derived from osteoblast-differentiated MSCs (min-ECM) had increased osteogenic potential than the other two. The different ECM compositions were the key regulators of the extremely different phenotypes with respect to mineralization. Therefore, comparative proteomic analysis of the ECMs was crucial to identify novel regulators of the molecular mechanisms underlying matrix mineralization. Of course, studying the behavior of cells on selected ECM components gives more straightforward results [32]. Moreover, biomaterials functionalized with synthetic biomimetic peptides have been designed [33]. However, cell-derived ECMs with their specific combination of proteins better represent the physiological microenvironment than a limited number of ECM proteins [34, 35]. For this purpose, proteomic studies are decisive to disentangle the ECM composition.

Mass spectrometry is a sensitive and high-throughput technique for the identification of many proteins within a single experiment. However, the large ECM proteins are very difficult to study, as they need to be carefully processed for MS as they are insoluble, highly glycosylated and cross-linked [36]. The protein composition of few cell-derived ECMs has been analyzed by MS [21, 37-39], but our knowledge about the complete protein composition is still limited. In **Chapter 2** we presented a comprehensive mass spectrometry analysis of the osteoblast derived-ECM, that revealed known structural ECM components, such as FN1, COL1A1, TNC within the most abundant proteins. The min-ECM displayed high homology (more than 50%) with the human bone proteome we previously analyzed [24], confirming the use of the devitalization treatment to produce a cell-derived ECM that reproduces an *in*

*vivo*-like microenvironment. Naba and coworkers classified the ECM proteome or 'matrisome' into core matrisome and matrisome-associated proteins and created an atlas of ECM proteins for an easier classification [37]. In addition to core matrisome proteins such as FN1, COL1A1, TNC, gene ontology analysis of our ECM revealed matrisome-associated proteins involved in matrix mineralization such as annexins (ANXA2, ANXA5, ANXA6) and ALP, explaining the role of the ECM in accelerating matrix mineralization. Interestingly, we also detected many mitochondrial and cytoskeletal proteins. We speculated that these proteins most likely arise from lysis of ECM-secreting cells, as mineralization is a high energy-demanding process with increased mitochondrial biosynthesis [40]. Moreover, cytoskeletal and apoptotic proteins were detected in the human bone samples [24]. Cell remnants from osteoblasts undergoing apoptosis are physiologically present in bone and regulate bone homeostasis and remodeling [41]. This highlights the supportive role of the ECM in reproducing a more complete microenvironment than just known proteins. Naba and coworkers set-up sequential steps to solubilize ECM components, followed by tandem quantitative mass spectrometry to study an *in-vivo* tumoral-ECM [37]. Recently, Ragelle used this technique to investigate the proteome of ECMs from bone marrow- and adipose tissue-derived MSCs [39], detecting similar matrisome components to our results by label-free quantitative strategies. Moreover, our results were in line with the other few groups that investigated the matrisome of cell-derived ECMs, though using different decellularization techniques and methods to counteract the ECM insolubility [16, 21, 38]. Our results contributed to disentangle the matrisome, but further work is needed to standardize protocols, toward the creation of a complete human ECM atlas of proteins.

We used MS to identify regulatory proteins that modulate bone formation, but of course interpretation of the data is essential. We and others used Gene Ontology and IPA analysis for data interpretation [16], though some ECM components are difficult to be accurately annotated and can be misassigned [42]. Interactomes can be built also by other software [43], that are constantly being improved towards the complete interaction networks of ECM proteins. For this reason, data re-analysis might be useful to achieve more insightful conclusions taking advantage of bioinformatic tools and database improvements over time. Of course, MS also gives unexpected or controversial findings. For instance, in our comparative proteomic analysis presented in **Chapter 3**, we were expecting to detect osteogenic inducers (such as BMPs) in the 149 proteins detected uniquely in the min-ECM, or inhibitors of mineralization (such as osteopontin) in the 31 proteins not detected in this ECM, considering the clear functional effect on mineralization. But this was not the case and in these 31 proteins we detected growth factors, such as TGF-β1 and CTGF, and proteins involved in vasculature development. These proteins were not directly

involved in regulating mineralization but more regulators of cell behavior and differentiation. Though very comprehensive, mass spectrometry gives a snapshot of a moment, and probably repeated measurements over time should have been performed for a complete overview. Unexpectedly, COL12A1, shown to promote mineralization, was upregulated in activin-ECM, in contrast with our functional data showing that Activin-ECM induced a lower matrix mineralization than min-ECM. On the other hand, inhibitors of mineralization such as AHSG, FETUB and ENPP1 were upregulated in the nonmin-ECM, along with our functional results showing that nonmin-ECM had a reduced osteogenic potential than min-ECM. We speculated that the entire ECM composition is important and most likely, osteogenic inducers must overcome the inhibitors of mineralization in the min-ECM, or maybe these proteins are differentially activated in the different ECMs. Overall, proteomic data need to be validated and accompanied by functional data, but are essential to identify targets for drug discovery or protein candidates to design bio-inspired and ECM mimicking substrates.

In **Chapter 2** and **3** we showed that ECM protein composition is essential to mediate the observed effects on cell behavior, due to the presence of proteins that promote cell adhesion, or that facilitate growth factor binding. In addition, also physical cues play an important role in the ECM-mediated effects on osteogenic differentiation. For instance, stiffness [44] or peculiar nanoscale surface topographies actively influence stem cell behavior [45]. In this respect, a more detailed analysis of the devitalized ECM stiffness, surface morphology, or the 3D structural organization of selected and labelled proteins, would help to disclose the ECM-cell interplay and use it for the rational design of bioinspired biomaterials for regenerative purposes.

## 6.4 HUMAN MESENCHYMAL STROMAL CELLS FOR BONE REGENERATION

Throughout this thesis, we mainly employed human bone-marrow derived MSCs, as they have been proposed and safely used as best candidates for bone tissue engineering, due to their osteogenic differentiation potential, the secretion of trophic factors and their immunomodulatory properties [46-49]. However, donor-to-donor variation, loss of the multi-lineage differentiation potential after *ex-vivo* expansion are still major limitations that need to be overcome [50, 51]. Of note, our experiments were performed on human bone marrow-derived MSCs, purchased by Lonza, but cells from different donors should have been tested to reinforce our results. Alternatively, MSCs from different sources than bone marrow, e.g. adipose tissue, have been proposed for the easier accessibility, holding great potential for bone tissue engineering applications (reviewed [52]). However proper bone formation *in vivo* by

these cells need to be assessed and distinguished from dystrophic calcification, as not many of them can induce hematopoiesis-supportive bone marrow stroma and vascularization [48].

In **Chapter 2** and **3** we showed that the osteogenic potential of MSCs was enhanced by growth on ECM compared to standard culture conditions *in vitro*. Furthermore, the percentage of proliferating MSCs was positively influenced by the ECM, as presented in **Chapter 2**. Assessing the maintenance of multi-lineage differentiation potential after passaging could represent a crucial step in order to use the ECM we created as alternative culture substrate to maintain MSC properties during cell *ex-vivo* expansion, as already proposed [11, 12]. However, *in vitro* results are not always confirmed *in vivo*. In some cases, the positive effect of cell-derived ECMs were not confirmed by bone formation *in vivo* [19, 53, 54]. In our case instead, we showed in **Chapter 2** that MSCs implanted with the devitalized osteoblast-derived ECM on HA led to ectopic bone formation *in vivo*, reducing the variation between different MSC preparations. The model of ectopic bone formation we used did not induce high amount of bone (Brum A.M *et al*, in preparation) [55], but was still sufficient to appreciate the differences induced by the presence and absence of ECM. When the ECM was not present, the amount of bone formation was variable, highlighting a role of the ECM in reducing the variation between different MSC preparations. A higher sample size would improve our study, as well as the investigation of vascular markers such as CD31, as proper vascularization to sustain the necessary growth factors is required for proper bone remodeling [56]. Moreover, a proteomic investigation of the composition of the ectopic bone formations would also be interesting to study in comparison with bone proteome, to analyze the potential enrichment of candidate proteins.

MSCs are still not routinely used for clinical applications. In view of using MSCs for bone tissue engineering, clinical grade facilities that allow *ex-vivo* expansion of MSCs while preserving their biological potential are needed to obtain high cell amounts required for clinical applications [57]. Accordingly, serum-free alternatives for MSC *ex-vivo* expansion should also be routinely used to meet good manufacturing practices (cGMP) criteria. In the context of bone tissue engineering, cell-based therapies seem more efficient than cell-free approaches, thus the combination of decellularized ECMs as scaffolds to be recellularized with live autologous cells represents an optimal combination [52].

## 6.5 ACTIVIN-A SIGNALING IN THE REGULATION OF ECM MINERALIZATION

Activin-A modulates several cell functions such as cell proliferation and differentiation, but its role in bone metabolism is still not fully clear [58]. Activin-A was previously shown to reduce matrix mineralization in osteoblasts [59-61]. In **Chapter 3** we showed that an ECM deposited by MSCs cultured with osteogenic inducers and in presence of Activin-A, still retained the Activin-A ability to reduce matrix mineralization, confirming our previous findings [61]. Proteomic data showed that BMP2 inducible kinase (BMP2K), known to impair mineralization *in vitro*, was the most abundant protein in activin-ECM. Moreover, many proteins were affected by the presence of Activin-A. Extracellular proteins such as COMP, HSPG2, FBLN2 were upregulated, whereas mitochondrial and membrane-proteins were downregulated in activin-ECM. Moreover, in **Chapter 4** we presented the effect of a 2-day treatment of Activin-A at early stages of osteoblast differentiation, that was able to reduce matrix mineralization at late stages of culture. This reinforces our previous findings in which human osteoblasts continuously treated with Activin-A exhibited a reduced matrix mineralization [59], and also shows the importance of timing of Activin A-presence during osteoblast differentiation on the subsequent effect on matrix mineralization. Interestingly, osteoblast gene expression profiles were modulated by Activin-A in a 2-wave fashion over time. In the first wave of gene expression changes, mainly genes involved in transcription regulation were affected, whereas in the second wave ECM proteins such as SPARC, OCN were modulated. ECM proteins were upregulated by Activin-A also in a human osteoblast cell line and in osteoblast-differentiated MSCs continuously treated with Activin-A [59, 61]. We speculated that Activin-A altered the ECM quality, without inducing a striking disruption, but having a mild but sufficient effect to alter matrix mineralization. Activin-A might stimulate the initial stages of osteogenesis and ECM maturation, but inducing an ECM composition that fails to support mineralization at later stages.

In **Chapter 4** we showed that TGF-β was upstream of the regulation of both waves of gene expression changes, and also genes regulated by Activin-A and Activin-A-modulated miRNAs were involved in TGF-β signaling pathway, highlighting the connection between Activin-A and TGF-β. The intricate cross-talk between Activin-A and TGF-β signaling pathways also involves SMAD2/3 phosphorylation [62]. TGF-β is stored in a latent form in the bone ECM and its availability is regulated by ECM proteins and osteoclast-mediated resorption [63]. TGF-β is known to stimulate the early stages of osteoprogenitor differentiation, but inhibits the late stages and matrix mineralization [64]. Activin-A also upregulated TGF-β gene expression in the second wave of gene expression changes, showing that differences in timing and in molecule bioavailability are crucial for the tight regulation of osteoblast differentia-

tion. However, due to the overlap between Activin-A and TGF-β signaling cascades it is also important to know the specific players of the signaling to specifically target the different pathways.

In **Chapter 4** we showed that Activin-A regulates genes involved in vasculature development, such as ANGPTL4, NOX4 and EDN1, in both waves of gene expression changes. Moreover, our findings showed that Activin-A also modulates miRNAs involved in erythropoiesis. As Activin-A acts as commitment factors for erythroid progenitors [65], studying the role of Activin-A in vasculogenesis is of particular relevance in fracture repair or control of bone tumor growth and metastasis.

Activin-A could be used as soluble molecule in case of ectopic bone formation or unwanted mineralization, though challenges remain to distinguish and specifically target pathological mineralization. However, studying how Activin-A modulates osteoblast gene expression, miRNA profile and ECM composition is important to identify potential targets involved in MSC commitment and thereby to modulate bone formation and bone quality.

## 6.6 OMICS APPROACHES FOR DATA ANALYSIS

Genome-wide (Omic) approaches are powerful tools to gain a comprehensive overview of the dynamic signaling pathways in the cell, which modulate their behavior in response to the extracellular microenvironment. Throughout this thesis, we took advantage of mass spectrometry to disentangle the proteome of different types of cell-derived ECMs (**Chapter 2** and **3**). We successfully used microarray data to investigate how Activin-A pulse influenced the transcriptome of osteoblasts (**Chapter 4**). And finally, in **Chapter 5** we employed the PamChip® array technology to investigate how cell adhesion to surfaces such as cell-derived ECM and titanium modulates the kinome profile of MSCs.

Kinases are key players in signal-transduction, regulating biological processes such as metabolism, cytoskeletal rearrangements for cell adhesion and migration, cell cycle progression and differentiation. The deregulation of kinase-mediated signaling leads to pathological conditions, thus making kinases ideal targets for cell therapy, such as by kinase-inhibitor drugs [66]. Kinase arrays are useful to study the kinome in physiological and pathological states, without *a priori* assumptions. Peptide array technologies have been successfully used to study different cell types in physiological conditions, such as LPS-stimulated cells [67], osteoblasts [68] and differentiation during hematopoiesis [69]. Moreover, kinase arrays were used to investigate pathological conditions to identify new target for clinical applications, such as in pediatric brain tumors [70], adenocarcinomas [71] and the impact of

osteoblast microenvironment on prostate cancer [72]. In **Chapter 5** PamChip results revealed that the kinomic signatures of MSCs adhering to the devitalized ECM and titanium were similar, but cells on ECM exhibited a higher level of active kinases, overall resulting in a higher phosphorylation. Moreover, we detected the activation of pathways such as FAK, Rho GTPase family and MAPK/ERK signaling, which are central hubs in modulating cell behavior upon integrin-mediated adhesion to a surface. Without *a priori* assumptions, we employed PamChip array to select PI3K/AKT signaling and further experiments confirmed the importance of PI3K/AKT signaling in MSC viability and adhesion. PI3K/AKT signaling is important also in osteogenic differentiation, and our preliminary experiments also confirm this finding (not shown).

Peptide arrays are based on the assumption that consensus sequences perfectly mimic protein phosphorylation *in vivo*. However, though the consensus sequences are literature-driven, they are not optimized for selectivity and software should be developed to improve substrate design [73]. Moreover, phosphorylation signal is a scale of intensities, thus setting up cut-offs and obtain statistical significance is still difficult. Peptide arrays guide hypothesis formulation, because the substrates can be phosphorylated by a variable number of putative upstream kinases [70, 74]. Thus, the array guides toward few pathways that need to be validated by traditional techniques, but bioinformatic programs need to be improved to functionally cluster the peptides to get significant and biologically relevant data. So far programs for DNA microarray analysis were used [67, 70, 71]. In our case, we took advantage of Markov analysis to determine the positive 'on calls' [75] that were further analyzed by IPA, but programs need to be implemented with more kinomic-oriented tools, especially to analyze the kinetic reaction over time and for functional clustering of peptides.

PamChip hold a great potential to tackle biological problems but also for therapeutic applications to enhance personalized medicine [66, 74]. Maybe the combination with other omic data will improve the investigation of biological problems by viewing the cell not only as separate compartments but as an intricate network of signaling pathways that relates with the surrounding microenvironment.



## 6.7 CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, our study highlighted the importance of the extracellular environment in modulating the behaviour of stem cells, giving important findings for bone biology and regenerative medicine. We showed the potential of our osteoblast-derived devitalized ECM in closely resembling the human bone tissue but especially enhancing the osteogenic potential of MSCs, both *in vitro* and *in vivo*. Our study

suggests that using native ECM may reduce MSC variations in *in vivo* applications, eventually improving the reproducibility and robustness of MSC clinical applications. The devitalized ECM could be used to coat scaffolds to create bioinspired biomaterials for bone tissue engineering applications, or be employed with MSCs in 3D printing technologies. The devitalized ECM was easily derived from MSCs, and these cells were the focus of our research due to their promising properties for bone tissue engineering applications. However, ECMs can be obtained from different cell types, revealing the high potential of cell-derived ECM applications. Moreover, the impact on several cell types could be investigated. For instance, the effect of the mineralizing ECM we presented is currently being investigated for its effect on endothelial cells, together with studies on the effect of hypoxia on the ECM composition. We modified the composition of the ECM and used proteomics and bioinformatic analyses to investigate the effect of the ECM on MSC osteogenic differentiation and mineralization, demonstrating that cell-derived ECMs are easily customizable. Patient-derived ECMs could be created to study pathologic conditions and impact on disease progression.

Moreover, we used microarray data to investigate the effect of Activin-A on matrix mineralization. Two-day treatment of Activin-A was able to alter osteoblast miRNA profile, as well as the gene expression in a 2-wave fashion over time, resulting in an alteration of the ECM compartment that failed to mineralize. The understanding of the mechanism of action of Activin-A could give further insights into implement the use of Activin-A to modulate MSC behaviour, that could be useful to regulate bone quality in case of unwanted mineralization of ectopic calcification.

Eventually, we also employed kinome array data and bioinformatic analysis to investigate how the ECM modulates the kinome profile of MSCs, and how this related to adhesion to titanium surface, already used for bone implants in clinical applications. We found that MSCs adhering to cell-derived ECM exhibited overlapping kinome profiles compared to cells on titanium, though with a higher kinase activation. We used kinase array to select PI3K/AKT signaling pathway without *a priori* assumptions, and confirmed its importance in MSC viability and adhesion. We illustrated that PamChip kinase array is a powerful technology to describe rapid changes in the kinase activities and their effect on the phosphoproteome, but also to investigate specific signaling pathways that can be targeted in the rational design of novel scaffolds, implementing the use of cell-derived ECMs for tissue engineering applications.

In the recent years, the ECM has gain interest, demonstrating to be not only an inert component of tissue architecture but an active modulator of stem cell behaviour. With this thesis, we contributed to disentangle the ECM-cell interplay for bone tissue engineering by creating cell-derived ECMs with different protein compositions

and direct the promising properties of MSCs for regenerative medicine. The ECMs represent useful tools to investigate protein candidates involved in mineralization, to regulate bone quality. Creating tailor-made ECMs by different cell types or patient-derived ECMs will broaden the applications of cell-derived ECMs to modulate stem cell behaviour or study disease progression, as well as to coat scaffolds for the rational design of bioinspired biomaterials for bone tissue engineering applications.

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