**Biosketch**

Nathalie Groen was born on 23rd September 1985 in Neerpelt, Belgium. She received her Bachelor of Science diploma in Biomedical Engineering from the University of Twente in 2008. Her graduation project investigated the tissue engineering application of PLA and PEG/PEI fiber based scaffolds. This project was performed under the supervision of Dr. Dominique Braemals at the Membrane Technology group of University of Twente. During early 2010, she graduated in Master of Science in Biomedical Engineering track Molecular and Cellular Tissue Engineering from the University of Twente. For her Masters project she investigated the osteoblastic differentiation potential of human mesenchymal stem cells at the department of Tissue Regeneration. Later, in 2010 she started her PhD training under the supervision of Prof. Jan de Beer and Prof. Carmen van Blitterswijk. Her doctoral research focused on unraveling the cellular response to varying biomaterials for bone tissue regeneration. This thesis describes the findings of her research work.

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**Invitation**

You are cordially invited to attend the public defense of my dissertation:

**TRANSCRIPTOME PROFILING TO UNRAVEL BIO-INSTRUCTIVE MATERIALS**

On Thursday, 20th of November 2014 at 12:45

In the Prof. dr. G. Berkhoffzaal Building De Waal University of Twente Drienerloaan 5 Enschede

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TRANSCRIPTOME PROFILING TO UNRAVEL BIO-INSTRUCTIVE MATERIALS

By

Nathalie Groen

2014
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Transcriptome profiling to unravel bio-instructive materials

Nathalie Groen

PhD thesis, University of Twente, Enschede, the Netherlands


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Cover Art: The combination of the binary code and the double-stranded helix represents the informatics analysis of gene expression, which is the basis of the research described in this thesis.

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TRANSCRIPTOME PROFILING TO UNRAVEL BIO-INSTRUCTIVE MATERIALS

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus

Prof. Dr. H. Brinksma

on account of the decision of the graduation committee,
to be publicly defended

on Thursday, November 20th, 2014 at 12.45 hours

By

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Table of Contents

Chapter 1

Introduction
1.1 Bone fracture healing
1.2 Synthetic biomaterials for bone regeneration
1.3 Outline of this thesis
1.4 References

Chapter 2

Introducing Materiomics
2.1 Introduction to Materiomics
2.2 The challenge of “living” materials science
2.3 Dealing with complexity
2.4 Emergence of Materiomics
2.5 Conclusion
2.6 References

Chapter 3

Bioinformatics-based selection of a model cell type for in vitro biomaterial testing.

Chapter 4

Exploring the material induced transcriptional landscape of osteoblasts on bone graft materials

Chapter 5

Distinct transcriptional profiles controlled by chemistry and surface topography in calcium phosphate ceramics

Chapter 6

High content imaging as a novel tool for automated analysis of biomaterial-induced cellular responses

Chapter 7

Reflections and future outlook: Considering complexities
7.1 Introduction
7.2 Classical biomaterial research
7.3 Complexity
Abstract

Spinal fusions and the repair of large bone defects resulting from trauma, tumors, infections or abnormal skeletal development are frequent surgeries in the clinic. These bone defects do not heal spontaneously and require grafts to bridge the defect, provide support to the surrounding tissue, and regenerate the missing bone. Although autografts are still perceived as the gold-standard for bone grafting, a wide array of alternatives available on the market has expanded, and research in search for improved alternatives has intensified in the last decade. In spite of the advances within this field of biomaterials for bone regeneration applications, the limitations in terms of material properties have not been solved and their mechanisms of action have yet to be elucidated. In this thesis I present a genomics approach to biomaterial research to help further advances within this field. The context and description of alternative bone graft substitutes are briefly summarized in chapter 1. Chapter 2 is a review dedicated to introduce materiomics as the holistic approach in materials research that considers a material as a complex system from which all the properties contribute to the overall (biological) capacity.

Developments in the field of biomaterials are often hampered by the lack of adequate in vitro models due to an incomplete understanding of in vivo mechanisms following biomaterial-cell and -tissue interactions. Therefore, in chapter 3, we defined a suitable cell type to study biomaterials in vitro by comparing and studying the transcriptional profiles of five different cell lines exposed to three well characterized biomaterials. Osteosarcoma-derived MG-63 cells were selected based on their ability to reflect the in vivo bone forming capacity of these three biomaterials in their transcriptome. This cell line was then employed throughout this thesis to study biomaterials with the aim of elucidating the instructive effect of biomaterials and biomaterial surfaces. To do so, we employed genomics tools and considered that the genome-wide transcriptional profiles illustrate the cellular state in response to the presented materials. As such, in chapter 4, we compared the cellular responses to various diverse, but commonly studied and clinically employed materials for bone regeneration applications in relation to material properties. Specific signalling pathways such as TGF-β and Focal Adhesion Kinase correlated to the bone forming capacity of biomaterials and are therefore hypothesized to play a role in material-induced bone regeneration in vivo. This specific correlation was further elucidated in chapter 5, in which we studied a selected subset of genes in relation to this bone-inducing property. The results not only confirm the complexity of biomaterials and their properties, but also point towards a role of the extracellular matrix composition for successful bone formation. Lastly, we compared in chapter 6 the transcriptional responses with morphological characteristics of cells when exposed to four materials with distinct surface roughness to explore the potential of high content morphological imaging as a tool to study, compare and screen biomaterials in a non-invasive manner. Chapter 7 includes final remarks discussing the envisioned approaches for successful developments in the field of biomaterials for (bone) tissue regeneration, to which the work presented in this thesis contributes.
Samenvatting

Wervelfusies en herstel van grote botdefecten, als gevolg van een trauma, tumoren, infecties of abnormale skeletontwikkeling, zijn veel voorkomende chirurgische ingrepen. De genezing van dit soort grote botdefecten gaat niet spontaan. Om het weefsel in en rondom het defect te helpen regenereren en om ondersteuning te bieden zijn daarom botvervangers vereist, die in het defect geïmplanteerd dienen te worden. Vooral nog is lichaamseigen bot nog steeds het meest gebruikte transplantaat voor reconstructies van dergelijke grote botdefecten en wervelfusies. Het afgelopen decennium is er echter ook steeds meer onderzoek gedaan naar alternatieve materialen en strategieën ter vervanging van dit lichaamseigen bot. Ondanks dat er een aantal effectieve materialen beschikbaar zijn op de markt, is het ook bekend dat deze materialen nog steeds hun tekortkomingen hebben. Met als gevolg dat de mechanische en biologische eigenschappen van deze biomaterialen nog steeds veel voorkomende en noodzakelijke onderzoeksonderwerpen zijn. In dit proefschrift presenteer ik een genomics-aanpak om de ontwikkelingen in het onderzoek naar biomaterialen verder te helpen. De context en beschrijving van alternatieve materialen voor botregeneratie doeleinden worden kort samengevat in hoofdstuk 1. Hoofdstuk 2 geeft een overzicht van materiomics als aanpak in het biomateriaalonderzoek, waarbij het materiaal als een complex systeem wordt beschouwd waarvan de combinatie van alle eigenschappen bijdraagt aan het algehele (biologische) gedrag van het materiaal.

Het gebrek aan adequate in vitro modellen als gevolg van onvolledige kennis van in vivo biomateriaal-gerelateerde mechanismen belemmert verdere ontwikkelingen. Daarom is om te beginnen een geschikte cellijn geselecteerd om biomaterialen in vitro te kunnen bestuderen (in hoofdstuk 3). We hebben de transcriptieprofielen van vijf verschillende cellijnen vergeleken en bestudeerd door ze op drie biomaterialen te kweken, waarvan de botvormende eigenschap in eerdere onderzoeken bewezen is. Uit deze geteste cellijnen zijn vervolgens osteosarcoma-afgeleide botcellen (MG-63) geselecteerd, doordat hun transcriptieprofiel de botvormende capaciteiten van de drie biomaterialen het beste weergaf. Vervolgens is deze cellijn toegepast in het onderzoek naar het effect van biomaterialen op gekweekte of omliggende cellen en weefsels. Ook hierbij hebben we gebruik gemaakt van de genomics-aanpak, door uit de transcriptieprofielen de celresponse op materialen af te leiden en in kaart te brengen. Dit hebben we in hoofdstuk 4 in de praktijk gebracht door uiteenlopende, veel bestudeerde en klinisch gebruikte materialen voor boeltersteltoepassingen te vergelijken en te relateren aan diverse materiaaleigenschappen. Hieruit kan onder andere geconcludeerd worden dat specifieke signaaltransductiewegen zoals TGF-β en Focal Adhesion Kinase correleren met de botvormende capaciteit van materialen. Deze correlatie tussen materiaaleigenschappen en genexpressie is verder uitgezocht in hoofdstuk 5. Hierin hebben we een specifieke subset van genen geselecteerd, waarvan de expressie duidelijk samenhangt met de botvormende capaciteit van materialen. De resultaten gepresenteerd in dit hoofdstuk wijzen in de richting van een rol van de extracellulaire matrixsamenstelling voor een succesvol botvormend materiaal. Tenslotte hebben we in hoofdstuk 6 transcriptieprofielen gekoppeld aan cel-morfologische eigenschappen door ze op vier materialen met verschillende oppervlakteruwheiden te kweken. Op deze manier kunnen we zien of cel-morfologische beschrijvingen kunnen worden toegepast om materialen
te testen op een niet-invasieve manier. De beoogde aanpak voor verdere succesvolle ontwikkelingen op het gebied van biomaterialen voor (bot)weefsel regeneratie, waar ook het in dit proefschrift gepresenteerde werk aan bijdraagt, worden tenslotte besproken in hoofdstuk 7.
Chapter 1

Introduction
Chapter 1

Introduction

Nathalie Groen
1.1 Bone fracture healing

Clinical relevance

Bone is a dynamic tissue and is subject to constant remodeling. Although the regenerative capacity of bone allows skeletal fractures to heal without complications, bone is, besides blood, the most commonly transplanted tissue. Significantly large bone defects do not heal spontaneously and require grafts to bridge the defect, provide support to the surrounding tissue and enhance bone regeneration. Indeed bone grafting is necessary for spinal fusions, to treat large bone defects and impaired fracture healing following post-traumatic complications. Spinal fusion, the most applied bone graft procedure, treats degenerative spinal conditions, severe back deformations or injuries. Significant bone defects result from e.g. tumor or infected and inflamed tissue resection or delayed-, mal- or non-union. Moreover, a significant amount of grafts are applied in maxillofacial surgery for periodontal defects and to fill bone loss associated with failed hip and knee arthroplasties.

Worldwide it is estimated that 2.2 million patients receive bone transplants each year resulting in a 2.5 billion dollar industry [1]. With a growing world population and an increased life-expectancy, it is fair to assume that these numbers keep on increasing, paralleled by an increasing demand for alternative clinical strategies to replace and repair damaged, degenerated and missing bone tissue.

Gold standard bone transplant

The gold standard in reconstructive orthopaedic surgery is autologous bone grafting. During the operation, bone is harvested from some part of the patient’s body, mostly the iliac crest, and shaped into the defect. It is successfully applied to treat non-unions in e.g. long bones, posterior cervical fusions and recalcitrant and infected non-unions. While filling the defect void, autologous bone grafts provide both mechanical support and biological components to drive new bone formation. The osteogenic precursors and osteoblastic cells surviving the transplantation compose the osteogenic potential. Moreover, the growth factors present in the autologous matrix, e.g. BMP2, BMP4, FGF and VEGF, drive cellular differentiation.

The biological effect of these grafts is the result of osteogenic, osteoinductive and osteoconductive properties. Osteoconduction refers to permitting the migration of bone forming osteogenic cells and the ingrowth of blood vessels and concomitant osteoprogenitor cells from the recipient surrounding bone tissue. Importantly, direct bonding of bone with the implanted material is promoted without the formation of fibrous tissue [2]. Osteoinductivity refers to the induction of undifferentiated osteoprogenitor cells that are not yet committed to the osteogenic lineage [3]. Osteogenetic refers to the presence of osteoprogenitors that are able to produce bone tissue [4].

Both cancellous bone and cortical bone are being used as autograft material. The choice naturally depends on the requirements as both have variable properties related to their
Introduction

structural anatomy. On the one hand, cancellous bone is highly porous and presents a much larger surface area with a higher number of diverse cell types (mesenchymal cells, immature and mature osteoblasts) resulting in better osteogenic, inductive and metabolic properties. Its porosity allows fast blood vessel ingrowth and concomitant influx of osteogenic cells and therefore a more rapid osseointegration. On the other hand, cortical bone is much denser and hence has better mechanical properties, providing structural support to the surrounding bone.

Unfortunately, harvesting autograft bone presents major drawbacks including the risk of donor site morbidity, infections, chronic pain, and, less frequently, neurovascular damage. It is not recommended for elderly, pediatric patients or patients with malignant or infectious diseases. Moreover, the availability of autologous grafts is a major concern. Another downside is the increased surgical time and hospital length with consequent additional costs. Importantly, the cellular components of the graft do not always survive the harvesting process while the osteogenic potential of the graft suffers inter-individual variability influenced by for instance genetic factors and age.

Alternative bone grafts

Alternatively, allografts are being used which are derived from donor bone (cadavers). Allografts are irradiated and stored frozen. Although allografts provide a calcium phosphate rich matrix, they lack osteogenic cells and proteins which hamper their osteogenic and osteoinductive potential. Besides their limited effectiveness, they also present potential disease transmission and immunogenic reactions.

Another form of commonly used allograft material is demineralized freeze-dried donor bone (demineralized bone matrix; DBM). Donor bone is demineralized and processed to reduce potential infections and immunogenic host responses while retaining its collagogenous structure. It is shown that the removal of the mineral phase renders the matrix more osteoinductive than the original mineralized allograft as it does not eliminate all the present growth factors. However, the mechanical properties of DBM are significantly reduced due to this decalcification step. The clinical outcomes of DBM remain inconsistent partially due to the differing proprietary processing methods of different suppliers influencing the remaining concentrations of growth factors [5-8]. Advantageously, DBM is supplied in varying forms, e.g. as a malleable putty, mouldable and injectable paste, chips or strips. It is widely used in maxillofacial regeneration and as a bone graft “extender” for skeletal regeneration rather than a substitute. The mechanical properties and variability of DBM grafts have promoted further research and development of synthetic graft materials.

Recombinant bone morphogenetic proteins are increasingly used for spinal fusions as alternative to auto- and allografts. OP-1 (rhBMP7, Stryker Biotech) and Infuse (rh-BMP2, Medtronic) are two products available on the market for orthopeadic applications. However, besides their considerable costs, major concerns exist on the effectiveness, high doses required during implantation and excessive tissue formation [9-11].

Moreover, collagen-based matrices from bovine origin are mainly used as composites with or carrier for other bone grafts. Collagen, as the main extracellular matrix
constituent of bone, contributes to mineral deposition, vascular ingrowth and growth factor binding providing a favourable environment to bone regeneration. Nevertheless, collagen provides minimal structural support. Bovine and coral derived hydroxyapatite is also available and used mostly in combination with a collagen carrier. The next section will discuss synthetic materials, their clinical history and potential in the context of bone grafting.

1.2 Synthetic biomaterials for bone regeneration

Within the field of biomaterials and tissue engineering, a tremendous body of work emerged on exploring and tailoring the physical and chemical properties of materials with respect to cell and tissue responses. Metals, ceramics, polymers and their composites have been studied and proposed as alternatives to autologous bone grafting. Synthetic materials are in unlimitedly supply, cheaper and easy to sterilize and store. Ideally, synthetic bone graft substitutes should possess numerous properties; as aforementioned osteoinductivity, osteoconductivity and osteogenicity are the key to support bone regeneration. Moreover, the bone graft substitute should be biocompatible, degradable, show minimal fibrotic reaction, allow remodeling, be macroporous to favor blood vessel ingrowth and osteogenic cells, and biomechanically stable. Importantly, the synthetic graft should provide initial stability, similar to autologous bone grafts.

Due to their similarities to the mineral phase of human bone calcium phosphate based materials present a great promise for bone regeneration applications. Early clinical success using a calcium phosphate compound to repair a bone defect was reported by Albee back in 1920 [12]. Calcium phosphates and bioactive glasses were the first bioceramics developed for bone repair. Ceramics are sintered (thermally heated) inorganic non-metallic solids. Although most calcium phosphate ceramics have osteoconductive and osseointegrative properties, some possess osteoinductive properties [13]. So far, because ceramic materials are considered mainly osteoconductive, they are used in small non-weightbearing applications (maxillofacial surgery) or as bone graft “extenders” rather than substitute materials. Tricalcium phosphate ceramic is the most osteoinductive ceramic and has been shown to have similar bone regenerative capacities to autografts and allografts in animal models [14, 15].

Nevertheless, calcium phosphate ceramics lack the required mechanical properties similar to the grafted bone even if the mechanical strength increases gradually with bone formation towards strengths similar to cancellous bone. Therefore, calcium phosphate ceramics were introduced in the clinic in the ’80s for minimal or non-loadbearing applications. Although considerable advances have been made with synthetic alternatives over the past decades and a diversity of materials with varying chemistries, fabrication processes and properties for diverse applications emerged, further developments have to be made on the e.g. osteoinductive capacity, mechanical properties and resorption rate. However, these developments are hampered as the evaluation of the bioactive properties of these materials is only reliable after implantation in large animal models due to the lack of adequate in vitro models.
1.3 Outline of this thesis

This thesis aims at contributing to the development of improved osteoinductive materials for bone regeneration applications. Developments in the field of biomaterials are often hampered by the lack of adequate in vitro models due to incomplete understanding of in vivo mechanisms following biomaterial-cell and tissue interactions. In order to acquire insights in the in vivo mechanisms induced by biomaterials, in vitro cell behavior was correlated to in vivo biomaterial performances. This correlation is central in this thesis and is presented as the approach to understand and unravel the interactions between cells and biomaterials. Importantly, the in vivo biomaterial performance is considered the result of the complex combination of separate material properties. Moreover, transcriptomics and bioinformatics analysis are the main tools used to characterize cell behavior and response to biomaterials.

Chapter 2 is a review dedicated to introduce “Materiomics” as the holistic approach in materials research that considers a material as a complex system that has to be studied as a whole. Within that line of thought, we first defined a suitable cell line to study biomaterials in vitro using bioinformatic analyses of transcriptional profiles. Osteosarcoma-derived MG-63 cells were selected based on their ability to reflect in their transcriptome the in vivo bioactive capacity of biomaterials which is a result of the complex combination of different properties (chapter 3). In chapter 4 we compared the transcriptional profiles of diverse, commonly studied and clinically employed materials for bone regeneration applications. In this chapter we map the different profiles which an osteoblast can adopt in response to materials with various both differing and overlapping properties. Correlating and comparing the materials allowed us to define testable hypotheses on the influence of material properties on gene expression. In chapter 5 we compared and combined the responses of osteoblasts and human mesenchymal stem cells to materials with osteoinductive capacities, circumventing various separate material properties, to elucidate the cellular mechanisms underlying this osteoinductive phenomenon. In chapter 6 we compared the transcriptional responses to morphological characteristics when cells were exposed to four materials with distinct roughness to point out the potential of high content morphological imaging as a tool to study, compare and screen biomaterials in a non-invasive manner. Chapter 7 includes final remarks discussing the envisioned approaches required for successful developments in the field of biomaterials for bone tissue engineering, to which the work presented in this thesis contributes.
1.4 References


Chapter 2

Introducing Materiomics
Chapter 2

Introducing Materiomics

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Chapter 2

2.1 Introduction to Materiomics

The ability to regenerate and repair tissues and organs – using science and engineering to supplement biology – continuously intrigues and inspires, hoping that the frailty of our bodies can be ultimately avoided. Ever since ancient times, surprising and unnatural materials have been used to (partially) substitute human tissues for medicinal purposes. For example, in the era of the Incas (c. 1500), molded materials such as gold and silver have been applied for the “surgical” repair of cranial defects. In addition, archaeological findings reveal a wide range of materials, such as bronze, wood and leather, used to replace and repair biological parts of the human body. Continuous refinement led to the first evidence of successfully implanted materials inside the body – reporting the repair of a bone defect in the 17th century.

Even earlier, the relationships between anatomy (i.e., structure) and function of living systems had been explored by Leonardo da Vinci and Galileo Galilei, one of the first few to apply fundamental science to biological systems. In the current age of technology, new materials for biomedical and clinical application have undergone a modern Renaissance, resulting in a surge of design and successful application [1-5]. The concepts of tissue repair and substitution are ever improving and becoming more accessible, proven by the widespread occurrence (and popular approval) of total hip and knee replacements, as examples. But rather than replacement with synthetic analogues, can biological tissue(s) be directly engineered? Admittedly, the first biomaterials arose to solve specific clinical problems and only later this became a field of research unto itself. It is apparent that polymers and ceramics (and other effective biomaterials) were not developed for implants per se – but rather were used because of their availability and proven (known) material properties. This need not be the case. The field of biomaterials has witnessed exciting and accelerating progression, partly due to the emergence of physical science based approaches in the biological sciences. Consequently, developments have led to a number of blockbuster materials which currently occupy a substantial part in modern healthcare with various clinical applications ranging from degradable intraocular lenses and sutures to coronary stents, heart valves and orthopedic implants. But ultimately, where does this field lead?

2.2 The challenge of “living” materials science

Hitherto, the field of biomaterials has largely been characterized by trial and error experimentation, practical intuition, and low throughput research [6]. As a direct result, identification and development of successful biomaterial candidates was frequently iterative – employing ad hoc, piece-wise, or one-off approaches to design and characterize materials for a specific application [7]. Currently lacking is a single set of “design parameters” which can satisfy more than the most rudimentary system – there is neither a standard “code” for biological systems nor a standard “toolset” for analysis.

In spite of continuous advancements in both the understanding of the natural function of biological materials and systems, as well as synthesis and regeneration of certain tissues (such as bone); a cohesive and systematic approach is still wanting. What is the primary impediment? Biological tissues, organs, and materials exploit multiple structures and functions across scales – they are universally hierarchical [8, 9]. Such
multi-scale hierarchies consequently make any single-scale analysis and prediction a hypothesis at best. While studies have successfully characterized components at specific scales (e.g., the molecular structure of DNA or the sequence of a multitude of proteins), superposition of the structure or the functional properties of individual components (defined differently according to scale) is insufficient to understand the complete system [10]. In simpler terms “1+1 ≠ 2”. We utterly fail in the “design” and “construction” of such material systems – we cannot accurately or reliably predict behavior of the final product. Indeed, whether a lack of critical system variables or understanding of system response, we are unable to model larger (living) multi-protein systems and networks, let alone the structural role such materials play in a cellular structure of tissue behavior. This is the exact opposite of the definition of engineering, where it is necessary to prescribe the performance of system components with reliable and repeatable accuracy.

Conversely, understanding the interaction of materials with biological (e.g., “living”) tissues across all scales – from atoms and molecules to tissues and eventually at the organism-level – remains a crucial hurdle in tissue engineering and biomaterial development. The challenge is intrinsically doubled-sided, yet highly intertwined. The scientific complexity at both sides of the interface – the material on the one hand and the organism on the other – needs to be considered (Figure 1). The fundamental problem of combining of living (biological) and non-living (synthetic) components can be encapsulated by the popular adage “the whole is greater than the sum of its parts” (commonly attributed to Aristotle, likely not referring to the interface of biology and materials). The complex interactions between materials and biological systems require certain tact to analyze deterministic (or predictive) behaviors and material properties. Nature, through meticulous trial-and-error and centuries of optimization and refinement, has intricately combined material structure, properties and functionality [8]. Structure and function are so intimately linked, even indistinguishable, that one-to-one substitution of other potential materials is currently not possible – but need this be the case?

**Figure 1. aA the interface of materials and biology.** The combination of living and non-living components - namely biological (represented by a human knee joint) and synthetic materials (represented by the building blocks) - present a complex challenge that can be summarized by the adage, ‘The whole is greater than the sum of its parts’. Here, the image shows the differential response of human mesenchymal stem cells (hMSCs) to different underlying topographies on the ‘TopoChip’ (Unadkat, Hulsman et al. 2011). Image courtesy of Frits Hulshof.
2.3 Dealing with complexity

Clearly, the concepts of Nature cannot be omitted from the equation when developing materials for biological applications. Evolutionary processes resulted in intricate biological systems, with robust and adaptable redundancies, as well as multifunctional and multiscale components, which set a hampering factor in compatible materials research – there are no material "standards" that all of biology must follow. This intrinsic complexity impedes full understanding and concomitantly limits developments in materials research for biological applications. Yet modern research has not sat idle, and has certainly provided us with the realization of the de facto complexity associated with biological systems. From a broad perspective, the causes of this complexity can be grouped into common categories: multi-scale; combinatorial and temporal (see Figure 2).

While the composition of biological materials is controlled by a relatively limited set of elements (i.e., carbon, hydrogen, oxygen, nitrogen and a few metal ions), this restriction is not imposed to biomaterials research (yet the laws and principles in materials science and chemistry remain applicable, allowing exploration beyond the confines of Nature). Nature is highly successful in creating diversity from this limited set of “building blocks” – such was indisputably demonstrated by the discovery of the structure of DNA by Watson and Crick in 1953, creating the illusion of a simple origin of life (only relying on four nucleotides). As a result, the fascination of growing any desired tissue from its basic DNA code, along with emerging expertise in (biological) material processing, became viable. One could easily foresee growing any desired tissue from the necessary DNA (along with requisite raw materials), similar to the chemical vapor deposition of carbon.

Figure 2. Sources of complexity in biological materials, with possible solutions via a materiomic approach.
Introducing Materiomics

nanotubes or the polymerization and spinning of nylon. It is merely the assembly of the appropriate “blocks”, so-to-speak.

Yet Nature revealed to be cleverer than that; evolution seamlessly intertwined structure and functionality. Despite protein materials being built, or “transcribed”, from a mere set of twenty amino acids, combinations of this limited set of building blocks endow a multitude of functionally distinct proteins [8]. Being said, proteins acquire their functionality across multiple scales via a combination of peptide sequence and common structural motifs (e.g., α-helices, β-sheets) and a set of prevalent processes and mechanisms (e.g., synthesis, breakdown, self-assembly) – the phenomenon of universality exists ubiquitously in biology. At higher scales, revealing the dimensions of biological complexity, proteins iteratively assemble into complexes; e.g., collagen fibrils, which in turn form collagen fibers and eventually assemble together with additional inorganic materials, are the major constituents of bone tissue. The structural conformation of proteins might be highly conserved throughout different tissues, while concurrently (and contrastingly) are highly tissue specific. A key starting point in developing working models for such complex systems is the preservation of particular functionality despite uncertainty or minor variation in components and/or the environment [10]. We must neglect the physical idiosyncrasies of a system (such as specific peptide sequence), identify the fundamental building blocks (e.g., structure, key interacting groups), and delineate the function of each (e.g., signaling, catalytic, mechanical, etc.). In essence, biological systems originate from their associated genomic sequence – a distinct sequence of simple base pairs. While true, such a description is as crude as describing Beethoven as a simple collection of notes or the works of Shakespeare as a linear sequence of letters [12-14]. The structural hierarchy and associated functionalities across scales add extra layers of complexity.

Another level of complexity arises from dynamic changes in biological material systems over time, owing to growth or adaption, for instance. To illustrate, the functional properties of proteins are also highly influenced by post-translational modifications (e.g., hydroxylations, phosphorylations, glycosilations) or enzymatic cross-linking. These modifications are crucial for the interaction with other proteins and material components and so determine the properties of tissues. At larger scales, cell adhesion, cytokinesis and cell migration illustrate the power of the cytoskeleton to self-organize locally into complex structures. Nevertheless, this complexity impedes understanding of biological processes as they are difficult to mimic or predict ex vivo or through synthetic approaches, posing a major challenge in structure prediction (and design) and the development of biocompatible materials. Simply put, biological materials grow (and/or evolve), while synthetic materials do not (i.e., characterized by static/constant material properties). It is apparent that not only are predictive models of assembly required, but also the possible development of self-adapting materials to mimic biological analogues.

Nature has creatively produced a broad range of functionally disparate materials (diversity) using a limited number of (universal) constituents, rather than inventing new building blocks. Therefore, such multi-scale hierarchical systems can simply not be analyzed or predicted at single-scale level. The so-called universality-diversity paradigm [15, 16] presents an alternative approach; it shifts the focus from individual component analysis towards the analysis of fundamental elements, hierarchical organization and
functional mechanisms (sometimes referred to as emergent properties, a concept common in the scope of systems biology). Yet again, “The whole is greater than the sum of its parts”.

But how can we (a) determine what function is required and (b) reduce the number of potential material candidates for our need? Two main approaches can be considered in materials research to deal with this complexity and understand and engineer biological systems. Firstly, a bottom-up approach: identify fundamental building blocks and study their structure, interactions and properties at all relevant scales, from Angstrom- to macro-level (from a single peptide to the collagen fiber). Secondly, via a high-throughput approach, study the biological roles of a material system as a whole (combining the best of holistic and reductionist approaches) [17-19].

Within the first perspective, investing in the relation between universal structures and corresponding functions is similar to the field of proteomics and interactomics [20-22], but extended beyond the confines of a cell and tissue to interactions and properties of materials. Observation and extraction of the general underlying principles (e.g., physical, chemical, optical, electronic, thermal, mechanical, etc.) of the structure-function relationship (using both experiments and theory) is required to make them available as concepts useful in materials science and engineering beyond biological occurrence, e.g., they should theoretically hold for similar synthetic material systems [23]. Being said, biological systems present inevitable complexity, introducing constraints in materials interactions analysis. Fields such as biomimetics attempt to exploit the structure and function (e.g., complexity) of such biological systems – applying principles of biology to synthetic systems – for the design and engineering of material systems [23, 24].

Continuing this line of thought – applying biological “tricks” to synthetic systems – we find that the problem quickly becomes intractable, as the shear amount of possible material-material interactions is unbounded. Moreover, unlike the biological limitation to available amino acids and ambient environmental conditions, in biomaterial research, complexity is further increased by the number of controls and variables produced by engineers (either by necessity or by choice). The second above mentioned approach, the employment of high throughput combinatorial methods, may provide the means to open-up new possibilities.

High throughput based methods allow simultaneous synthesis/processing and evaluating of a multitude of system variations (e.g., material, molecular) [25] to isolate desired behavior/responses. Such methods were commonly applied within the field of pharmacology for drug discovery [26], the successful genetic screening of fruit flies and zebra fish [26], and variegated applications in systems biology [27], as examples. Building on these past successes (also including proteomics or genomics [28, 29]), modern approaches have accelerated the discovery process and analytical methods, and have likewise extended insights and potential applications. Far from autonomous improvement, successful studies rely on the technological advancements of many fields, as every step involved in this approach requires high throughput methods; from synthesis characterization (e.g., from a chemical or structural perspective) to analysis and characterization of the desired outcome (e.g., at cellular or tissue level) [30].
Introducing Materiomics

The screening process is relatively simple: when the awaited performance is attained (based on a variety of metrics), a suitable material or system candidate can be defined, and subsequently iterated. With following reductionist investigation, one can impinge on the relation between “universal” material components and observed biological response, such as the relationship between surface chemistry or topology and a biological phenomenon of interest such as cell differentiation. As such, unraveled pathways and mechanisms may serve as basis for further material refinement and development. Advantageously, no theoretical background of complex biological processes is required a priori to screen for performance of material systems – only the results drive the screening process. As such, critical performance metrics and material properties may unexpectedly emerge upon characterization and analysis of successful outcomes, leading to new insights and target parameters. Such a holistic screening of systems together with reductionist characterization of the phenomenon can be reciprocally beneficial, providing a self- optimizing protocol for delineating material system characteristics and performance, beyond the scope of any one-off system investigation. High throughput screening of a material property within a specific application can lead to unexpected or even unnatural findings which can in turn lead to optimized design of new materials.

Recently, in spite of the discussed intrinsic biological complexity, the advances in (biological) material sciences are considerable. Indeed, continuous refinement of techniques provide new, more accurate means to measure, interpret, quantify, and model the relationships between chemistry, structures, design and function. Progress in information technology, imaging, nanotechnology and related fields – coupled with developments in computing, modeling and simulation – have transformed investigative approaches of materials systems. The motivation has come from a vast assortment of disciplines: medicine (physiological properties of tissues for prosthetic devices, replacement materials, and tissue engineering applications); biology (material aspects of adaptation, evolution, functionality, etc.); and materials science (thermal and electrical properties of nanosystems, functional performance of microscale devices, etc.), as examples. Undoubtedly, both the potential reward and challenge of the understanding of biological materials elicits contributions from biologists, chemists, and engineers alike. Further advancement is hindered, however, by such a “divide and conquer” approach, and dictates a convergence of scientific disciplines under a common banner – a.k.a. materiomics.

2.4 Emergence of Materiomics

Traditionally, materials science, in its broadest sense, has assembled distinct research areas based on classes of structures, length scales and varying functionalities (structural, thermal, electronic, etc.). Indeed, there is a co-existence of disparate disciplinary affiliations, such as the specialties of ceramics and polymers, the fields of nano- and microtechnology, or the area of bioactive materials for distinct and specific applications. In Nature, however, reciprocal refinement (e.g., evolution) has led to a balance between chemistry, materials, structure and required function. From this perspective, the disciplinary boundaries in material sciences should be razed, and the merger (or convergence) of different disciplines is inevitable. The rich history, experience and unique perspectives of distinct fields in combination with their diverging approaches, technological advancements and methodologies promote altruistic progress in this
inherently interdisciplinary venture (Figure 3). Unsurprisingly, combining the widespread acquired knowledge and expertise of materials scientists with the detailed understanding of biological systems and structures build over years by biologists, holds great promise.

The emerging field of materiomics works from this philosophy of convergence, and is characterized by a materials science approach that considers all mechanisms of a material system across multiple scales. Materiomics – the transparent combination of “material” with the suffix “omics” – is most simply defined as the holistic study of materials systems. Materiomics represents a necessary holistic approach to biological materials science (systems with or without synthetic components), through the integration of natural functions and biological processes (i.e., “living” interactions) with traditional materials science perspectives (e.g., physical properties, chemical components, hierarchical structures, mechanical behavior, etc.). The suffix -omics, with reference to similar fields with this holistic approach like proteomics or metabolomics, emphasizes the complexity of such study. The term omics, by definition, refers to “all constituents considered collectively”. In comparison, genomics is defined as the study of the human

![Figure 3. Materiomics - the convergence of disparate fields.](image)

The interface of materials science ("synthetic") and biology ("life") has been successful in the development biomaterials, but recent technological advancements (computational capabilities, experimental methods such as AFM, imaging techniques such as NMR) allow for a truly integrated and holistic approach. While some biological materials have been investigated from a materials science approach, and some material developments have been inspired by Nature, complete understanding requires integrated and holistic approaches. One direction has been to uncover the functional relationships of biological materials (e.g., physiological function through proteomics attained via bioinformatics) while another direction systematically characterizes the material properties of tissues via modelling and experimental probes common to materials science (e.g., mechanistic interpretations of function derived from molecular simulation). Materiomics resides at the apex of these information streams, attempting to reconcile biological function with material interactions and properties.
Introducing Materiomics

genome referring to all the genes of the considered organism and not just a small subset of genes that determines the observed phenotype. Equally, materiomics entails much more than the commonly used approach of piece-wise unraveling the properties and behavior of a material. It entails the study of all possible functionalities and properties in a holistic fashion. For example, the process of bone tissue growth on a calcium phosphate scaffold under controlled conditions is a materials science and biological problem (albeit nontrivial). Understanding completely how bone tissue can be grown on any arbitrary material platform is a materiomics problem. At the juncture is the emergence of the materiome, which can be thought of as the abstract collection of all material behaviors/functions/interactions with all potential material systems and environmental conditions.

Innovation and successful (predictive) biomaterial design involves a rigorous understanding of the properties and mechanisms of biological matter. Consequently, the understanding of the necessity to merge fields, as well as attempts of combining fields of “biology” and “materials science”, are being currently undertaken without the widespread adoption of the term “materiomics”, resulting in continued advancements in research on complex biological and synthetic material systems. Indeed, while biological materials appear challenging and irreducibly complex, advancements in biomaterials synthesis and self-assembly are far from sitting idle. Conceivably, and necessarily, several “spin-off” research areas emerged to satisfy the needs to allow progression in materials research driven by this new approach. Indeed, biologically “themed” interdisciplinary research areas originated such as bioinformatics, nanobiology or systems biology (see Figure 3). Through the merging of technologies, processes and devices into a unified whole, new pathways and opportunities for scientific and technological progressions are created inaccessible to any single discipline or knowledge base.

The current knowledge and advanced technology, acquired over years by material scientists, enabled the production of large material libraries (“living” and/or synthetic) with diverse chemical properties [31]. As such, libraries based on block co-polymers chemistry [32, 33], or click-chemistry [34-37], or surface topography have emerged encompassing as well the cataloguing of protein databank (http://www.rcsb.org/). These libraries are of interest for the materiomics approach in biomedical material science. Assembly of such libraries is obviously crucial for the progress in this field; however, one must realize that the existence of material data should be distinguished from material knowledge. While assembly of material libraries is important (and a necessary step), it is moot without associated understanding of material function – it is akin to filling a library with a diversity of books, yet being unable to read a single word. Such a book collection is a striking yet severely oversimplified metaphor, indicating the assembly of materials only represents the first steps in materiomics-based material development, just as determining the genome sequence is the first step in unlocking the power of the genetic code. Importantly, the analysis of material properties with respect to its biological and functional influence is the variable to address, as inspired by Nature, where the structure and function of a system are intimately interlinked. Equally, slight alterations in underlying chemistry of a biological system may have great influence on its resulting functional properties, and may serve as inspiration or guideline in further materials development.
2.5 Conclusion

The field of materials research for biomedical and clinical applications has witnessed an exciting development over the past several years. Herein, an ‘omics approach has been undertaken and is forecasted to guide the field to develop and progress at higher and more efficient rates. Indeed, from the materiomics perspective, the field of biomedical materials research has to rely on a holistic approach to investigate biological material systems. As most material properties are strongly dependent on the scale of observation, integration of multi-scale experimental and simulation analyses are the key to improve our systematic understanding of how structure and properties are linked. Therefore, convergence of different scientific fields with their distinct knowledge and methodologies is necessary to tackle the challenges of this holistic ‘omics approach. It is apparent that, at the interface of living and non-living materials, the “the whole is greater than the sum of its parts”. Understanding of such complex systems, therefore, requires more than the summation of disciplinary contributions – fields and techniques must be integrated in a cohesive and synergistic manner (a cooperative “whole”). Further streamlining of the process from material banking to assay development, high content imaging and data mining will ascertain that the materiomics approach will become available for the biomaterial research community.
Introducing Materiomics

2.6 References

Bioinformatics-based selection of a model cell type for in vitro biomaterial testing
Chapter 3

Bioinformatics-based selection of a model cell type for in vitro biomaterial testing.

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Abstract

Biomaterial properties can be tailored for specific applications via systematic and high throughput screening of biomaterial-cell interactions. However, progress in material development is often hampered by the lack of adequate in vitro testing methods, frequently due to incomplete understanding of involved in vivo mechanisms. In line with drug discovery in pharmacology, a crucial step in assay development for biomaterial screening is the identification of a target to direct the screen against. Herein, the cell type to be used for screening is of essential importance and has to be carefully chosen. So far, few attention has been put on selecting a cell type specifically suitable for in vitro testing of materials for predefined applications. In this manuscript, we describe an approach to define a suitable cell type for screening assays, for which biomaterials for bone regeneration served as example. Using a bioinformatics methodology, different cell lines are compared on three well characterized model materials. The transcriptional profiles of MG-63, iMSC, SV-HFO, hPPCT, hBPCT and SW480 cells are assessed on 3 calcium phosphate-based materials to evaluate their potential application in a screening assay. We show that MG-63 is the most suitable cell line to evaluate biomaterials for bone regeneration applications, evidenced by their robust gene expression differences between the 3 model materials. The gene expression differences between the cell lines were assessed based on the overall gene expression profiles and specific subsets of genes and pathways related to osteogenesis and bone homeostasis in response to the 3 materials tested. In the next phase, this cell line will be used to identify a target correlating with in vivo biomaterial performance and henceforth to develop an in vitro screening system.
3.1 Introduction

The need for tissue reconstruction keeps on inspiring the design of new materials and grafts that support the body during the healing process. As such, biomaterials represent a significant part of modern healthcare with numerous clinical applications ranging from vascular grafts, intraocular lenses and degradable sutures to orthopaedic implants. In general, biomaterials aim at achieving adequate biological interaction with the surrounding living tissue of the host upon implantation to replace damaged parts. It is believed that the biomaterial surface properties dictate the biological response both in vivo and in vitro [1]. However, progress in material development within diverse biomedical fields may be hampered by the lack of adequate in vitro testing methods partially due to incomplete understanding of involved in vivo mechanisms [2].

The pharmacological field, and more specifically the process of drug discovery may serve as an exemplary strategy in the development of in vitro models to screen materials. Essentially, drug discovery involves the identification of a target against which a therapeutic compound has to be directed. Using DNA microarray technology hits are identified which are typically confirmed by functional interference experiments on specific cell lines. If corresponding gene targets give rise to predicted phenotypes in knockout mice, the hit is used as a target for drug discovery. In effect, employing a candidate approach, the target is typically used in a high-throughput screening (HTS) system wherein large libraries of biological or synthetized chemical compounds are tested on their ability to bind, modify or inhibit the target function. Emerging hits are then confirmed on their specificity by cross-screening other targets. Subsequently, these hits are characterized and validated in several runs of in vitro and in vivo biological testing assays and models. With reference to this HTS methodology, several platforms and methods have been developed to screen in vitro material properties and cell-material interactions using cell-based readouts [3]. 2D as well as 3D screening assays in both array or gradient designs have been used to test the influence of different material parameters including surface chemistry (i.e. wettability, presence of functional groups), surface topography, patterns, microstructures, material porosity, natural or synthetic material composition (ECM proteins, polymer blends), matrix elasticity, etc [4-10]. In addition, libraries of polymers, DNA sequences, small peptides, soluble signals such as growth factors and trace elements evolved to identify hits and evaluate their influence on cell behavior [11]. Typically, adhesion, proliferation, differentiation and metabolic activity of the cells are used as readout for material characterization, and specifically for bone regeneration applications the readout is frequently limited to alkaline phosphatase (ALP) activity, matrix mineralization or the expression of markers like OC or RUNX2 [9, 12, 13]. However, the correlation between the currently used in vitro markers and corresponding in vivo performance remains often to be proven. In contrast, in vitro models to test haemocompatibility of materials using platelet adhesion as negative readout are commonly accepted [14, 15].

Within the field of calcium phosphate based materials, major developments resulted in increasing clinical applications for skeletal regeneration (i.e. dental implants, orthopedic implants). Originally, their similarities to the mineral phase of human bone made calcium phosphates interesting biomaterial candidates. Besides, they are considered osteoconductive and some possess osteoinductive properties[16-18]. Despite this
promising bioactive behavior, the use of ceramics in the clinic remains limited to implant coatings and small sized low-weight bearing applications due to their weak mechanical support, brittleness and low tensile strength. Therefore, the mechanical properties of ceramics are still subject of research and progress has been made by for instance combining calcium phosphates with polymer blends [19, 20]. In effect, the mechanism underlying bone healing upon calcium phosphate grafting is not fully understood. Therefore, the evaluation of the bioactive properties of these materials is only reliable after implantation in large animal models due to the lack of adequate *in vitro* models. To date, biomaterials exist and can be produced with a multitude of diverging biological and functional properties for a wide range of biomedical applications. Moreover, with the growing knowledge and developments in the chemical, biological and mechanical field, materials and material properties keep on being subject to research. Logically, in order to upscale the evaluation and consequently the improvement of materials there is a need for alternative *in vitro* screening methods. Progress in the field of microfabrication enabled technologies such as high-throughput assays to identify genome wide gene expression of cells. Using this technology, nearly 50,000 potential genetic markers covering the whole genome are tested simultaneously. In search for an *in vitro*

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**Figure 1. Study outline.** The bone forming capacity of three ceramic particles, HA, BCP and TCP, was previously characterized ectopically (shown schematically on the left side by the red basic fuchsin staining and the graphical representation). In search for an *in vitro* model to test this bone forming property of materials, different cell lines are tested on their ability to reflect material-induced cellular changes (schematically represented on the right side). In effect, we cultured six different cell lines on the three model materials and the corresponding gene expression profile was assessed using DNA microarray.
model, this technique offers more potential hits compared to a candidate approach on a subset of molecular markers. So far, this technique has been used to compare gene expression profiles between different cells, cell sources, treatments, tissues, etc [21-23]. To our interest, it has been specifically used to assess the best cell type, cell source or donor for implantation. However, it has never been used, to our knowledge, as a tool to identify the best cell type to characterize or screen biomaterials in vitro. In our opinion the cell type to be used in screening assays is crucial and has to be carefully chosen, as the biological effect is species-, source- or donor-site specific [24-28]. In effect, we previously showed that a small molecule (dibutyryl-cAMP) is capable of modulating the secretome of human MSCs [27] and its osteogenic differentiation capacity whereas it induces a different phenotype in MSCs derived from other species [28]. An adequate in vitro model is often believed to mimic in vivo environment, however, the cell types involved in vivo bone regeneration upon graft implantation are not defined. Bone marrow derived mesenchymal stromal cells (hMSC), blood vessel derived pericytes, osteoblasts, osteoclasts are suggested to play a role [29, 30]. More specifically, we found the expression profile of a few genes correlating with the in vivo osteoinductive capacity of these materials [16]. In addition, hMSC have been shown to be able to respond to different osteogenic stimuli [12]. However, it is of crucial importance to emphasize their high donor variability, phenotypical instability of passaging and their very mild response to osteogenic stimuli (for instance BMP-2) [31]. In our lab, we previously demonstrated the response of hMSCs to different calcium phosphate ceramics [16], but also to Wnt agonists [32, 33] and compounds which interfere in collagen crosslinking [34]. In all these cases, the number of differentially expressed genes was low, and the fold changes were modest. From this perspective, hMSCs are not an eligible cell type for screening assay development. Therefore, we evaluated the robustness of the response of 6 different cell lines and hence their eligibility to be applied in a screening assay. More specifically, we analyzed their material-induced sensitivity by comparing their transcriptional profile when seeded on 3 well studied and characterized calcium phosphate ceramic materials. We included hydroxyapatite (HA), biphasic calcium phosphate (BCP) and tricalcium phosphate (TCP) which are known to have different osteogenic and bioactive capacities [16]. The outline of the study is represented Figure 1.

3.2 Materials and methods

Cell Culture

A colorectal adenocarcinoma derived epithelial cell line (SW480, ATCC; kindly provided by Prof. H. Clevers), a peripheral nerve derived pericyte cell line (hPPCT) and a blood-brain barrier derived pericyte cell line (hBPCT, both kindly provided by Prof. T. Kanda) [35] were cultured in basic medium composed of Dulbecco's modified Eagle's medium (DMEM, Life Technologies), 10% fetal bovine serum (FBS, Lonza), 100 U/mL penicillin (Life Technologies) and 100 g/mL streptomycin (Life Technologies). Both pericyte cell lines were expanded on collagen type I coated 100 mm plates (BD BioCoat™) at 33 °C. The osteosarcoma cell line MG-63 and immortalized mesenchymal stromal cell line (iMSC, kindly provided by Dr. O. Myklebost (Oslo University Hospital, Norway)) were expanded in basic medium containing α-minimal essential medium (α-MEM, Life Technologies), 10% fetal bovine serum, 0.2 mM ascorbic acid (Asap, Life Technologies),
Cell selection for in vitro biomaterial testing

2 mM L-glutamine (Life Technologies), 100 U/mL penicillin, 100 µg/ml streptomycin. A human pre-osteoblastic cell line (SV-HFO; [36, 37]) was cultured in basic medium containing phenol-free α-MEM (Life Technologies), 10% FBS, 100 U/mL penicillin, 100 µg/ml streptomycin, 20 mMol HEPES and pH-adjusted to 7.5. Medium was refreshed twice a week and cells were used for experiments, further sub-culturing or cryopreservation upon reaching near confluence. Further experiments were performed at 37 °C and 5% CO₂ for each cell type.

Cell Type Characterization; Mineralization and Calcium Assay

To characterize the different cell lines, their mineralization capacity was determined. The cell lines were seeded in T25 flasks at 5,000 cells/cm² and cultured in mineralization medium for four weeks. For SW480, iMSC and both pericyte cell lines, mineralization medium consisted of their respective basic medium supplemented with 10 ng/mL dexamethasone (Dex, Sigma) and 0.01 M β-glycerophosphate (BGP, Sigma). Mineralization of SV-HFO cells was tested in basic medium containing 2% FBS supplemented with 100 ng/mL Dex. MG-63 cells were tested for mineralization capacity in basic medium containing 8 mM calcium (CaCl₂·2H₂O, Sigma) [38]. As a negative control, cells were cultured in basic medium supplemented with 0.01 M BGP. The total calcium deposition as a result of mineralization was analyzed using the QuantiChrom™ Calcium Assay Kit, according to manufacturer’s protocol. Briefly, 0.5 N HCl was used to release calcium overnight and the calcium content was measured at 620 nm.

Synthesis and Characterization of Calcium Phosphate Ceramics

Hydroxyapatite (HA) ceramics were prepared from HA powder (Merck) using the dual-phase mixing method and sintered at 1,250 °C for 8 h according to a previously described method [39]. Biphasic calcium phosphate (BCP) ceramics were fabricated using the H₂O₂ method using in-house-made calcium-deficient apatite powder and sintered at 1,150 °C (BCP) [17]. The method used to synthesize the BCP ceramics was also used for preparation of tricalcium phosphate (TCP). TCP ceramics were prepared from TCP powder (Plasma Biotal) and sintered at 1,100 °C. Ceramic particles (1–2 mm) were prepared, cleaned ultrasonically with acetone, 70% ethanol and demineralized water, dried at 80 °C, and sterilized by gamma irradiation prior to use.

The macro- and microstructure of the different ceramics was evaluated using a SEM (XL30, Environmental SEM-Field Emission Gun, Philips).

Cell-Material Characterization

To characterize the cell-material constructs, 200,000 cells were seeded per standard volume of 150 µL of pre-wetted material in 100 µL basic medium and allowed to attach for 4 h before adding additional medium. The cell-material constructs were cultured in their respective basic medium (without any additives) for 48 h, after which cross sections were made to evaluate the cell distribution throughout the material. To this
end, cells were fixed on the materials in 10% formalin for 20 min, dehydrated with 70% ethanol and embedded in methyl methacrylate (MMA, LTI) for sectioning. Sections were processed on a histological diamond saw (Leica SP1600). Sections were etched with an HCl/ethanol mixture and sequentially stained with 1% methylene blue (Sigma) to visualize the cells and their distribution.

The viability of cells cultured on the constructs was assessed visually by a Live-dead assay (LIVE/DEAD® Viability/Cytotoxicity Kit, Invitrogen). After 48 h of culture, the cell-material constructs were rinsed with PBS and stained with calcein AM/ethidium homodimer using the Live-dead assay Kit, according to the manufacturers' instructions. The viability of the cells was evaluated using fluorescence microscopy; living cells fluoresce green and the nuclei of dead cells fluoresce red.

Gene expression profiling

To investigate which cell type is most susceptible to changes in its gene expression profile instructed by different materials, cells were seeded onto the materials as described above. However, for iMSC, initial seeding number was increased to 300,000 in order to obtain sufficient RNA to perform the microarray study. After 48 h, total RNA was isolated from each cell type cultured on the materials using TRIzol (Invitrogen) and the Nucleospin RNA isolation kit (Macherey-Nagel) according to the manufacturer's protocol. Then, from 350 ng of RNA, cRNA was synthesized using the Illumina TotalPrep RNA amplification Kit, according to the manufacturer's protocol and both RNA and cRNA quality were verified on a Bioanalyzer 2100 (Agilent). Microarrays were performed using Illumina HT-12 v4 expression Beadchips, according to the manufacturer's protocol. Briefly, 750 ng of cRNA was hybridized on the array overnight after which the array was washed and blocked. Then, by addition of streptavidin Cy-3 a fluorescent signal was developed. Arrays were scanned on an Illumina Beadarray reader and raw intensity values were background corrected in BeadStudio (Illumina). Further data processing and statistical testing were performed using R and Bioconductor statistical software [40]. Graphical visualization of the data was obtained using GeneSpring software (Agilent Technologies Inc. and Strand Life Sciences Pvt. Ltd.). The probe-level raw intensity values were quantile normalized and transformed using variance stabilization (VSN). A linear modelling approach with empirical Bayesian methods, as implemented in Limma package [41], was applied for differential expression analysis of the resulting probe-level expression values. $P$-values were corrected for multiple testing using the Benjamini and Hochberg method [42]. Genes were considered differentially expressed between TCP and HA when a corrected $p$-value below 0.05 was reached.

Furthermore, the genes whose expression pattern over the 3 materials correlated with the osteogenic capacity of these materials to form bone at ectopic sites in mice [16] were quantified or more specifically, genes whose expression throughout the 3 materials have similar (positive or negative) trend as the different amounts of bone induced by HA, BCP and TCP, which is 0%, 17.7 ± 5% and 28.7 ± 4.8% respectively. This correlation was determined using Pearson's correlation coefficient and genes with a correlation $p$-value below 0.01 were considered.
The microarray data were subjected to multinomial regression to analyse the association between gene expression and the type of material on which the cells are cultured, using the R package GlobalTest [32]. All tested probes (47,231) were included in a regression model and the tested null hypothesis was that the expression of genes was not associated with the type of material. This analysis approach was not only applied to all probes, but also on a specific subset of genes known to be related to bone. This reduced list of 1244 genes was generated using Endeavour online software [33], a computational approach to prioritize candidate genes related to the following set of genes and pathways: BMP2 (ENSG00000125845), IBSP (ENSG00000029559), ALPL (ENSG00000162551), SPP1 (ENSG00000118785), SPARC (ENSG00000113140), PMF1/BGLAP (ENSG00000160783), RUNX2 (ENSG00000124813), WNT signalling pathway (Kegg:04310) and BMP signalling pathway (GO:0030509). This gene list was prioritized using functional annotations (retrieved from Gene Ontology and KEGG databases), biomolecular or protein interactions networks (BIND and STRING databases) and text mining data based on scientific literature and own experience. Furthermore, specific GO and KEGG pathways (listed in table 4) of interest were tested for their association with the gene expression using the Global test correct with Holm's multiple testing method.

Statistics

All experiments were performed in triplicates and as the mean ± standard deviation. Assays were analyzed using Student's t-test or using ANOVA with Tukey post-hoc testing comparing all groups of interest. Statistics used during bioinformatics characterization are explained in detail in subsection 2.6.

3.3 Results

Characterization of cell types studied

Establishing a highly specific cell-based screening system to test in vitro the osteogenic potential of materials requires a well characterized assay and an elaborated selection of the cell type. Therefore, 6 different human cell lines were chosen to be evaluated on their behavior and response to 3 diverse and well-characterized ceramic materials. The choice for these cell lines was based on their potential involvement in material-induced bone formation, i.e. cells attracted by virtue of the biomaterial and responsible for bone formation.

To characterize and compare the selected cell lines, a long term differentiation study was performed and the cell lines were specifically tested on their ability to mineralize, i.e. deposit calcium on their extracellular matrix indicating osteogenic differentiation potential. After 4 weeks of culture in mineralization medium, the amount of calcium deposited as a result of mineralization was quantified. MG-63, SV-HFO, iMSC and hBPCT showed extensive calcium deposition as compared to the corresponding negative control, whereas neither SW480 nor hPPCT were able to produce a mineralized matrix (Figure 2).
Characterization of materials used for cell type selection

Three calcium phosphate ceramics were included in this study to test the genetic response of the different cell lines; hydroxyapatite (HA), biphasic calcium phosphate (BCP) and tricalcium phosphate (TCP). The macrostructure of the 3 different ceramic materials did not differ from each other, as illustrated by scanning electron microscopy (Figure 3 A, D, G). Higher magnification images illustrate the difference in microstructure in terms of pores and grain size. The grain size is smaller and pore incidence higher in TCP and BCP compared to HA (Figure 3).

Characterization of cell-material constructs

Figure 4 A illustrates the distribution of MG-63 throughout the ceramic construct, also visualized by methylene blue staining on cross-sections (Figure 4 B and C). Cells are sparsely and evenly distributed over the whole construct without the formation of cell...
Cell selection for in vitro biomaterial testing

clumps. The effect of the material on the cells is optimized by the surplus of material surface compared to attached cell surface in order to ensure maximized cell-material interaction. Additionally, life dead assay revealed that the majority of the cells distributed throughout the construct appeared viable (green staining in Figure 5).

Material Induced Gene Expression Profiling

Next, we isolated RNA from the cells cultured on the materials after 48 h, excluding unattached and death cells, performed a microarray study and determined the genetic differences induced in the cell lines by the 3 different materials tested. Principal Component Analysis (PCA) allows visualization of the correlation between the different samples based on differences in their whole genome expression profile. The genetic profile of the different cell types when cultured on the materials reveals the similarity between both pericyte cell lines, as shown in the PCA plot (Figure 6). This similarity confirms the sensitivity of the DNA microarray technology used. The intrinsic differences between the cells surpass greatly the genetic differences induced by the materials. Moreover, SW480 are genetically more distinct from the other cell types as indicated by the distance between the samples on the PCA plot.

Figure 4. Characterization of the cell distribution on the materials. A: Representative overview image of a methylene blue stained MG-63 cultured for 48 h on BCP (scale bar 100µm). B and C: methylene blue stained cross-sections of the same construct (scale bar 100 µm and 50 µm respectively).

Figure 5. Viability of the cells cultured on the materials. Representative images of viability assay performed on MG-63 cultured for 48 h on HA (top row), BCP (middle row) and TCP (bottom row). The materials are covered with green dots (images left column), representing the viable cells, whereas only very few red stained death cells (images right column) could be observed (scale bar 500 µm; applies to all images).
Evaluating gene expression profiles: unbiased approach

The cell line best suited to characterize materials in vitro is selected based on its sensitivity to cues provided by materials, i.e. the cell line that reveals most genetic difference in response to materials. Previously, we and others have demonstrated that the 3 materials used in this study as model system, induce ectopic bone tissue formation to various extents in the muscle of large animals [16, 17] and mice [43], also referred to as osteoinduction. To recapitulate, HA does not induce bone tissue formation whereas BCP implantation results in 17.7 ± 5% and TCP in 28.7 ± 4.8% of bone apposition per scaffold area, as assessed previously in sheep [16]. Ideally, the cell type used to characterize materials in vitro should reflect these clearly dissimilar in vivo results found, and therefore we evaluated the correlation between the genome-wide expression values from the different cells to the quantified bone tissue formation results. First, the overall expression profiles appeared to vary markedly on the different materials for most cell types (Table 1, middle column). Indeed multinomial logistic regression (assessed by Global Testing [44]) revealed that MG-63, iMSC and hBPCT have a general expression profile that differs on the tested materials, indicated by a p-value below 0.01. The expression profiles of SW480 and the hPPCT cell line were less influenced by the type material used, having a p-value below 0.05. The SV-HFO cell line was not influenced when cultured on the

Figure 6. Genetic expression profiles of different cell lines cultured on the 3 materials. Principal component (PC) analysis to characterize the gene expression profiles from the different cell lines.

<table>
<thead>
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<th>P-value</th>
<th>P-value</th>
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</tbody>
</table>

Table 1. Association of the gene expression profiles of the different cell types with the 3 tested materials. (Middle column) Regression analysis revealed the overall expression profiles (all probes tested by DNA microarray) varied considerably on the different materials. Specifically, the expression profiles of MG-63, iMSC, hBPCT (p < 0.01), SW480 and hPPCT (p < 0.05) was significantly associated with the type of material. (Right column) Regression analysis revealing the significance (p-value) of the association between the expression profiles of a bone-related subset of genes and type of material.
different materials ($p=0.107$). Next, we assessed the amount of genes with an increasing or decreasing expression value between HA, BCP and TCP, reflecting the \textit{in vivo} bone formation results as reported above. The amount of genes whose expression correlated ($p < 0.01$) with the tendency in bone formation volume was considerably higher in MG-63 and iMSC than in SV-HFO, hPPCT, hBPCT and SW480 (Table 2). MG-63 cultured on the 3 materials revealed 1167 genes with an expression changing gradually from HA to BCP and to TCP. For iMSC, a comparable amount of 1094 genes correlated with this osteoinductive material property. SV-HFO only showed 594 correlating genes, hPPCT 744, hBPCT 786 and the epithelial SW480 cell line 715.

Furthermore, aside from the amount of genes correlating with the osteoinductive capacity of the 3 materials, we assessed the amount of genes differentially expressed between HA and TCP, both extremes in terms of \textit{in vivo} osteoinductivity. Again, we observed that MG-63 and iMSC had substantially more changes in their genetic profile compared to the other studied cell lines (Table 3). In fact, 1465 genes were significantly (corrected $p$-value $< 0.05$) differentially expressed between TCP and HA in MG-63 whereas iMSC showed 1406 genes differentially expressed. SW480 revealed only 209 genes differentially expressed, SV-HFO only 115, hBPCT 107 and hPPCT 80 genes.

<table>
<thead>
<tr>
<th>Cell type</th>
<th># genes with Correlation $P &lt; 0.01$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-63</td>
<td>1167</td>
</tr>
<tr>
<td>iMSC</td>
<td>1094</td>
</tr>
<tr>
<td>SW480</td>
<td>715</td>
</tr>
<tr>
<td>SV-HFO</td>
<td>594</td>
</tr>
<tr>
<td>hPPCT</td>
<td>786</td>
</tr>
<tr>
<td>hBPCT</td>
<td>744</td>
</tr>
</tbody>
</table>

\textbf{Table 2. Correlation of the genetic expression profiles of the cell lines with the osteoinductive property of 3 materials.} Quantification of the genes whose expression pattern correlated with the factorial osteoinductive property of the materials. The factorial osteoinductive property of HA, BCP and TCP is 0, 1 and 2 respectively. The correlation between expression of a gene and the osteoinductivity was calculated based on Pearson's correlation coefficient. Genes exposing a correlation $p$-value lower than 0.01 were considered in the quantification.

<table>
<thead>
<tr>
<th>TCP - HA</th>
<th>$P &lt; 0.05$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-63</td>
<td>1465</td>
</tr>
<tr>
<td>iMSC</td>
<td>1406</td>
</tr>
<tr>
<td>SW480</td>
<td>209</td>
</tr>
<tr>
<td>SV-HFO</td>
<td>115</td>
</tr>
<tr>
<td>hPPCT</td>
<td>80</td>
</tr>
<tr>
<td>hBPCT</td>
<td>107</td>
</tr>
</tbody>
</table>

\textbf{Table 3. Differentially expressed genes between TCP and HA.} Quantification of the genes significantly (corrected $p$-value $< 0.05$) differentially expressed when comparing TCP with HA.
Evaluating gene expression profiles: Biased approach

In addition to quantitative evaluation of the expression differences evoked by the materials in the tested cell lines, we qualitatively evaluated these differences. In order to test the response of the cells in a more specific fashion, we narrowed the gene list to test down from approximately 47,000 probes to 1,244 genes which have a relation to the process of bone formation. Using a bioinformatics tool, we produced this narrowed gene list by assembling genes related to the following candidate genes and pathways: ALPL, OC, OP, WNT and BMP signaling. Next, multinomial logistic regression was performed on this subset of genes to evaluate the association between their expression and the type of material (Table 1, right column). This analysis demonstrated that the expression of the bone-related subset of genes in iMSCs, MG-63 and hBPCT is significantly associated with the type of material (p < 0.01). Again the response of SW480 and hPPCT was less, but still significantly, associated (p < 0.05). In contrast, the association p-value between the expression of genes in SV-HFO remained insignificant. Both the above-mentioned findings indicate that gene expression of MG-63, iMSCs and hBPCTs is affected in response to the 3 materials.

Next, the expression of genes assembled in all GO categories and KEGG pathways was tested for association with the three materials using multinomial logistic regression (with the type of material as a categorical variable). However, we could not identify bone-specific terms and pathways in the top lists of the different cell types tested. Therefore, we then assessed the association between the expression of pathways and ontology terms relevant to osteogenesis or bone homeostasis (retrieved from KEGG and GO databases), and the type of material (Figure 7 and Table 4). Genes involved in bone specific pathways and gene ontology terms, such as osteoclast differentiation, BMP, WNT, hedgehog and VEGF signaling are differentially affected in MG-63 and iMSCs when cultured on the different materials. Genes annotated with ossification are associated with the type of material in nearly all cell types except for hPPCT. Additionally, we tested 3 randomly chosen pathways (PPAR signaling, Glycosaminoglycan biosynthesis – keratin sulfate and primary bile acid biosynthesis) which were in general not specifically affected by the

<table>
<thead>
<tr>
<th>KEGG / GO term</th>
<th>MG-63</th>
<th>iMSC</th>
<th>SW480</th>
<th>SV-HFO</th>
<th>hPPCT</th>
<th>hBPCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0001503 Ossification</td>
<td>0.0050</td>
<td>0.0038</td>
<td>0.0198</td>
<td>0.0226</td>
<td>ns</td>
<td>0.0052</td>
</tr>
<tr>
<td>KEGG:4380 Osteoclast differentiation</td>
<td>0.0238</td>
<td>0.0210</td>
<td>ns</td>
<td>ns</td>
<td>0.0458</td>
<td>ns</td>
</tr>
<tr>
<td>GO:0030509 BMP signaling pathway</td>
<td>0.0050</td>
<td>0.0116</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.0051</td>
</tr>
<tr>
<td>KEGG:4310 Wnt signaling pathway</td>
<td>0.0202</td>
<td>0.0348</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>KEGG:4340 Hedgehog signaling pathway</td>
<td>0.0238</td>
<td>0.0348</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>GO:0004991 Parathyroid hormone receptor activity</td>
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<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>KEGG:4370 VEGF signaling pathway</td>
<td>0.0238</td>
<td>0.0276</td>
<td>ns</td>
<td>ns</td>
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<td>ns</td>
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<tr>
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<td>ns</td>
<td>ns</td>
<td>0.0366</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>KEGG:533 Glycosaminoglycan biosynthesis - keratin sulfate</td>
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<td>0.0078</td>
<td>ns</td>
<td>ns</td>
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<td>ns</td>
</tr>
<tr>
<td>KEGG:00120 Primary bile acid biosynthesis</td>
<td>ns</td>
<td>ns</td>
<td>0.0110</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 4. Association between the expression of genes from specific gene sets of interest and the type of material. This table provides the association p-value (corrected for multiple testing) for each gene set of interest for the different cell lines together with 3 randomly chosen gene sets (bottom 3 rows of the table).
type of material. However, the genes involved in GAG biosynthesis exhibited a different expression pattern in iMSCs on the materials, which was also true for genes involved in PPAR signaling and primary bile acid biosynthesis in SW480.

Overall, MG-63 and iMSCs respond better to the cues provided by the materials in terms of gene expression. This was shown both in a quantitative and qualitative approach.

### 3.4 Discussion

The development of new synthetic bioactive materials for bone defect reconstruction is hampered by the lack of adequate in vitro testing models and poor insights in material-induced bone formation. Indeed, osteoinductivity, an important inherent biomaterial property for bone regeneration purposes in critical-sized defects, can mostly be verified solely in large animals like goats and dogs and not in less expensive small laboratory animals such as mice. As correctly stated by Yuan et al. [45], the biomaterial field is now forced to develop more suitable substitute models that eventually also support the understanding of the biological processes involved in bone regeneration. As early as 1983, Brown specified the need for standardized in vitro testing of materials for biomedical applications [46]. Indeed, in our opinion, we should in general aim at predictive tools for in vitro evaluation of the (in vivo) bioactive performance of materials. As such, a wide range of samples may be tested on their bioactive outcome without prior knowledge of specific underlying biological processes.

![Figure 7: Association between the expression of genes from specific gene sets of interest and the type of material. This graph represents the association p-value (corrected for multiple testing) for each gene set of interest for the different cell lines together with 3 randomly chosen gene sets (last 3).](image)
Different cell lines are widely used for the characterization and evaluation of biomaterials for biomedical and orthopaedic applications. However, different cells show different outcomes, as evidenced by Vohra [24], where the adhesion and spreading on HA particles of 2 osteosarcoma cell lines was mediated via different proteins than in hMSC. Moreover, the osteoblast like cell line MG-63 is well-characterized to study the properties of materials in vitro (e.g., the study of microstructured titanium surfaces for bone formation applications [47-49]), specifically by assessing osteoblast adhesion, proliferation, differentiation and typical marker expression. Although a tumor cell line, MG-63 are relatively immature osteoblasts with bone forming osteoblastic traits. Similarly, primary mesenchymal stromal cells (hMSCs), derived from various tissues but principally from bone marrow, are widely used to evaluate the in vitro bone formation ability of biomaterials. However, the use of cell lines derived from hMSCs, like iMSC used in this manuscript, is rare [50]. ALP and OC are proteins related to osteoblast function whose expression is used for in vitro testing even if conflicting results between in vivo and in vitro evaluation exist. Furthermore, mineralization of ECM is also related to osteoblast function and similarly, it is used as a read out to test biomaterials in vitro.

Target identification for biomaterial research, i.e. bone regeneration applications, seems seldom as simple as within disease prevention and recovery. Efforts for unravelling biological processes underlying biomaterial-induced bone regeneration were so far not successful. With this manuscript we have undertaken the first step in identifying targets predictive for in vivo performance of biomaterial-induced bone formation, which is defining an appropriate cell line to use. A few requirements were stated prior to cell type decision; the quantity of gene expression differences between the materials, the specificity of the expression responses and the amount of genes which expression correlates with osteoinductive property of the materials observed in vivo. Among the different cell lines tested, MG-63 and iMSC fulfilled the criteria set the best. Nonetheless, in view of developing a high throughput screening assay, the need of increased cell numbers for RNA isolation points towards bad iMSC attachment and growth on biomaterials and is therefore a less suitable cell line.

The DNA microarray technology employed to determine the cell type has shown to be reliable and reproducible. This is confirmed within this work by the observed clustering of the replicate samples and the clustering of the two pericyte cell lines which are developmentally more similar compared to the other cell lines. The next step in our approach to establish an in vitro model for biomaterial screening is the identification of diagnostic markers able to predict the in vivo outcome. The key is the correlation between in vivo evaluation of biomaterials with in vitro parameters like for instance gene expression. Inherently, genetic markers which expression may be used for in vitro evaluation of biomaterials may also eventually provide fundamental insights in the underlying biological processes and pathways induced by material-cell interactions involved in material-induced in vivo bone formation. To our knowledge, this type of approach has never been applied to identify suitable cell types for in vitro material testing for predefined applications.
3.5 Conclusion

The data presented in this article describes the approach taken to define a suitable cell type in order to establish an adequate and reliable model for in vitro testing of biomaterials which is widely lacking at present. Using a bioinformatics approach we have shown that MG-63 is the most suitable cell line to evaluate biomaterials for bone regeneration applications.
3.6 References

Cell selection for in vitro biomaterial testing


Chapter 4

Exploring the material induced transcriptional landscape of osteoblasts on bone graft materials
Chapter 4

Exploring the Material Induced Transcriptional Landscape of Osteoblasts on Bone Graft Materials

Nathalie Groen, Niloofar Tahmasebi, Fumitaka Shimizu, Yasuteru Sano, Takashi Kanda, Davide Barbieri, Huipin Yuan, Pamela Habibovic, Clemens A van Blitterswijk and Jan de Boer

Abstract

During the past decades, there have been a number of major advances in the field of biomaterials, thereby generating a vast variety of materials for a broad range of tissue engineering and regeneration applications. Although gene expression profiling has been used occasionally in biomaterial research, its usefulness for understanding cell-biomaterial interactions should be further explored for it to fulfill its promise as a tool to assess and improve material properties. Here we explore the transcriptional landscape induced by a variety of material properties within the scope of bone regeneration. An osteoblast cell line was used to identify the gene expression profiles that osteoblasts can adopt in response to biophysical and chemical cues provided by materials. The gene expression profiles induced by 23 different materials uncovered molecular and cellular responses that help us understand cell-biomaterial interactions while confirming the usefulness of transcriptional profiling in biomaterial research. As such, we show that TGF-β and WNT signalling are involved in the cellular response to osteoinductive materials along with differential cell adhesion kinetics via attenuated FAK signalling. We confirmed the previously reported effect of calcium and phosphate on BMP2 and TGF-β signalling. Moreover, the presence of nano-hydroxyapatite in poly (D,L-lactic acid) polymer particles affects the metabolic and translational state of the cells, which is abolished when the surface of these composites is etched. Together with future applications, this approach will help researchers understand cellular responses in relation to material properties, which will promote the development of more effective biomaterials for applications in tissue regeneration.
4.1 **INTRODUCTION**

The material properties dictate biological responses *in vitro* and *in vivo*. Understanding these cell-material interactions have been subject to extensive research in the last decade. A body of work has emerged on the modulation of cell morphology and behaviour by material properties such as surface roughness, topography, chemistry and elasticity (1-3). Increasingly, instead of assessing cellular responses to individual material properties or material candidates, investigations have adopted a high-throughput approach with the emergence of libraries of varying chemistries, topographies and stiffness for instance (4-8). Combinatorial libraries were also designed to investigate the multifactorial effect of material properties (9-11). With these developments, the biomaterials field has begun to undertake a high-throughput materiomics approach to address the biological effects of material properties (12).

The characterization of cellular responses to various materials and properties has been mainly focused on cell adhesion, proliferation, self-renewal, migration and differentiation. Indeed, only a limited number of studies have attempted to unravel the biological mechanisms preceding the observed functional readouts. While mostly low-throughput techniques such as quantitative RT-PCR and western blotting have been used, microarray profiling allows the investigation of the biology underlying the cellular responses to material properties in a high-throughput fashion. Historically, this technique has shown its usefulness for investigating biological signalling pathways, studying mechanisms involved in development, disease and cell niches, and in investigating the effect of therapeutics in pharmacology (13-19). Gene expression microarray technology allows a global measurement of nearly all known protein-coding genes. Within the context of biomaterials research, the cellular responses to different surface structures have been investigated using gene expression profiling (5, 20-22). Also, gene expression and networks were analyzed on ceramic/collagen composites, electrospun and spin-coated materials, and different ceramics in order to understand the cellular response to the presented physico-chemical stimuli (23-25).

We previously observed an effect of different ceramic materials at the gene expression level and we have shown that gene expression profiling can provide insights in molecular mechanisms involved in the phenotypical effects (25-28). In this study, we report the cellular response to a set of 23 varying materials using a genomics approach. These materials include a wide variety commonly used in the context of bone repair and regeneration such as ceramics, demineralized bone matrix (DBM), composites and metals. The biological effect of the majority of these materials has been investigated *in vitro* (e.g. cell proliferation and osteogenic differentiation) or *in vivo* (e.g. bone forming capacity). Based on transcriptional profiles, we correlated material properties to biological responses (*i.e. in vivo* osteoinductive capacity and known molecular mechanisms) in order to understand the molecular effects of cell-biomaterial interactions. This study shows a genomic approach to study material properties in relation to their biological effect.
4.2 Materials and methods

Materials

Various materials considered relevant for bone regeneration applications were used. These materials were collected from different providers (Table 1). Detailed production and fabrication methods from the manufacturer are described in the supplementary information. Briefly, eleven calcium phosphate-based ceramics were obtained from two different suppliers: Cam Biomaterials B.V. (Leiden, The Netherlands) and Xpand Biotechnology B.V. (Bilthoven, The Netherlands). These ceramics consisted of three types of hydroxyapatite (HA; HAa, HAb and HAc), two types of β-tricalcium phosphate (TCP; TCPa and TCPb) and five biphasic calcium phosphates (BCP; BCPa-e), comprising a mixture of HA and TCP. The eleven ceramics differed in physico-chemical properties as a consequence of differences in raw material and fabrication process. Five different composite materials were included based on poly (D,L-lactic acid) (PLA) blended with nano-sized HA powder, etched with increasing concentrations of NaOH (0, 0.125, 0.25, 0.5 and 1 M; PCa-e). In order to study the effect of adding a ceramic component to a polymer, we also studied one PLA, one PLA coated with a thin layer of octacalcium phosphate (OCa).

<table>
<thead>
<tr>
<th>Name</th>
<th>Materials</th>
<th>Supplier</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAa</td>
<td>HA – Ts=1150°C</td>
<td>Xpand Biotechnology BV</td>
<td></td>
</tr>
<tr>
<td>HAb</td>
<td>HA – Ts=1250°C</td>
<td>Xpand Biotechnology BV</td>
<td></td>
</tr>
<tr>
<td>HAc</td>
<td>HA – Ts=1100°C</td>
<td>Cam Biomaterials BV</td>
<td></td>
</tr>
<tr>
<td>TCPa</td>
<td>HA/TCP (80/20) – Ts=1150°C</td>
<td>Xpand Biotechnology BV</td>
<td></td>
</tr>
<tr>
<td>TCPb</td>
<td>HA/TCP (80/20) – Ts=1300°C</td>
<td>Xpand Biotechnology BV</td>
<td></td>
</tr>
<tr>
<td>TCPc</td>
<td>HA/TCP (80/20) – Ts=1150°C</td>
<td>Cam Biomaterials BV</td>
<td>Composite</td>
</tr>
<tr>
<td>TCPd</td>
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<td>Cam Biomaterials BV</td>
<td></td>
</tr>
<tr>
<td>TCPe</td>
<td>HA/TCP (60/40) – Ts=1150°C</td>
<td>Cam Biomaterials BV</td>
<td></td>
</tr>
<tr>
<td>TCPf</td>
<td>β TCP – Ts=1050°C</td>
<td>Xpand Biotechnology BV</td>
<td></td>
</tr>
<tr>
<td>TCPg</td>
<td>β TCP – Ts=1100°C</td>
<td>Cam Biomaterials BV</td>
<td></td>
</tr>
<tr>
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<td>PLA</td>
<td>PLA – OCP coated</td>
<td>CaP coating</td>
</tr>
<tr>
<td>PLAC</td>
<td>PLA</td>
<td>PLA/nCaP (50 wt.%) – 0 M NaOH</td>
<td>Xpand Biotechnology BV</td>
</tr>
<tr>
<td>PCA</td>
<td>PLA</td>
<td>PLA/nCaP (50 wt.%) – 0.125M NaOH</td>
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<tr>
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<td>PLA</td>
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<td>Xpand Biotechnology BV</td>
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<td>PLA</td>
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<td>Xpand Biotechnology BV</td>
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<tr>
<td>PCIe</td>
<td>PLA</td>
<td>PLA/nCaP (50 wt.%) – 1M NaOH</td>
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</tr>
<tr>
<td>bECM</td>
<td>Decellularized trabecular bone</td>
<td>EMCM BV</td>
<td></td>
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<tr>
<td>DBM</td>
<td>Demineralized bECM</td>
<td>EMCM BV</td>
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<tr>
<td>Ti</td>
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<td>Ti-OCa</td>
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<tr>
<td>PS</td>
<td>Polystyrene</td>
<td>Nunc</td>
<td>Polymer</td>
</tr>
</tbody>
</table>

Table 1. Overview of the 23 materials included in this study that were subjected to transcriptional profiling in osteoblasts. The supplier and the type of material are listed in the 3rd and 4th column. Detailed fabrication processes and descriptions of these materials are provided in the supplementary information.
phosphate OCP (PLA-OCP) and a composite based on this PLA blended with nano-sized HA (PLAC). Also, titanium foam was included (Ti), uncoated or coated with a OCP film (Ti-OCP) as used for the PLA. Two autograft chips were included with two different treatments: decellularized (bone extracellular matrix; bECM) and decellularized and demineralized trabecular bone (demineralized bone matrix; DBM) obtained from EMCM B.V. (Nijmegen, the Netherlands). All materials were provided in particles of 1 to 2 mm in size, except for the two titanium scaffolds which were 5 mm in diameter and 10 mm in length. Tissue culture polystyrene (PS; Nunc) was included as a reference material.

The surfaces of the 23 materials were characterized using scanning electron microscopy (XL30, Environmental SEM-Field Emission Gun, Philips) coupled to energy dispersive X-ray analyzer (EDAX; Apollo X, Ametek).

Cell culture

The osteosarcoma cell line MG-63 was cultured in medium consisting of α-minimal essential medium (α-MEM, Life Technologies) supplemented with 10% fetal bovine serum, 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin and 100 µg/ml streptomycin. Medium was refreshed twice a week and all cell-material experiments were performed at 37 °C and 5% CO₂.

To investigate the effect of the materials on MG-63, 200,000 cells in 100 µL medium were seeded per 150 µL of material pre-wetted with medium, and allowed to attach for 4 h before adding additional medium to the well. The cell-material constructs were cultured for 48 h or 7 days. As a reference, MG-63 were seeded at 5,000 cells per cm² on tissue culture polystyrene and cultured for the same time periods.

Transcriptional profiling

RNA isolation
Total RNA was isolated using a Nucleospin RNA isolation kit (Macherey-Nagel) according to the manufacturer’s protocol. Then, from 275 ng of RNA, cRNA was synthesized using the Illumina TotalPrep RNA amplification Kit (Ambion), according to the manufacturer’s protocol. Both RNA and cRNA quality were verified on a Bioanalyzer 2100 (Agilent).

Gene expression profiling
Microarrays were performed using Illumina HT-12 v4 expression Beadchips. Briefly, 750 ng of cRNA was hybridized on the array overnight, after which the array was washed and blocked. Then, by addition of streptavidin Cy-3, a fluorescent signal was developed. Arrays were scanned on an Illumina Beadarray reader and raw intensity values were background corrected in BeadStudio (Illumina).

Gene expression analysis
Further data processing and statistical tests were performed using R and Bioconductor
Material-induced transcriptional profiles

statistical software (29). The probe-level raw intensity values were quantile normalized and transformed using variance stabilization (VSN). T-distributed Stochastic Neighbor Embedding (t-SNE) was applied using the ‘tsne’ implementation in R (30). A linear modeling approach with empirical Bayesian methods, as implemented in the Limma package (31), was applied for differential expression analysis of the resulting probe-level expression values. P-values were corrected for multiple testing using the Benjamini and Hochberg method (32). Significant differential expression was considered for corrected p-values below 0.05. Gene lists were assembled using Endeavour online software (33), a computational approach to prioritize candidate genes related to a set of genes and pathways based on annotations retrieved from the KEGG database and biomolecular or protein interactions from the Bind, Biogrid InNetDb, Intact, Mint and String databases. This was applied to obtain a list related to the BMP2 gene, resulting in 425 genes or 651 illumina probes. Another list of 932 molecules (1503 probes) related to the 90 genes involved in TGF-β signaling was extracted from the KEGG database (KEGG:04350). KEGG analyses were performed in R supported by the ‘clusterProfiler’ package. The lists containing genes related to osteoinductivity were generated in two ways. The first list assembled all genes significantly differentially expressed between the osteoinductive (HAa, BCPa and TCPa) and non-osteoinductive (HAb and BCPb) materials. The second list was based on the genes whose expression correlated, using Pearson’s correlation coefficient, with the quantitative osteoinductive potential (i.e. the amount of bone that the materials form when implanted intramuscularly).

Ingenuity Pathway Analysis (IPA, Qiagen) was used to evaluate the signalling and metabolic pathways overrepresented in the differentially expressed genes. The overrepresentation was calculated using the right-tailed Fisher’s exact test adjusted using the Benjamini-Hochberg multiple testing correction (pathways with corrected p-values below 0.05 were considered).

4.3 RESULTS AND DISCUSSION

In order to map the material-induced molecular responses elicited in cells, the transcriptional profiles of MG-63 cultured on a set of 23 different materials were studied and compared. The materials, summarized in Table 1, are commonly used or studied as potential bone substitute materials. In order to understand the molecular mechanisms induced upon interaction with the biomaterial, early gene expression variations were assessed. As such, the gene expression profiles induced by different calcium phosphate ceramics, polymers and composites thereof, titanium, ceramic coated surfaces, demineralized (DBM) and decellularized bone matrices (bECM) were compared after 48 h. An osteosarcoma-derived osteoblast cell line (MG-63) was cultured on the materials, after which the transcriptional changes were assessed. We have previously shown that this cell line, commonly used in biomaterials research, is highly responsive at the gene expression level to ceramics with varying surfaces (34). In the work reported here, the biological effects of the materials are studied with the aim to demonstrate the use of transcriptional profiling in investigating cell–material interactions and cellular responses to materials depending on their properties.
General Transcriptional differences induced by materials

General observations
DNA microarrays assess the expression of over 47,000 probes, simultaneously representing nearly all the protein-coding genes (over 20,000) from the human genome. In order to explore the general expression variations induced by the different materials (i.e. the similarities and differences between the gene expression profiles), principal component analysis (PCA) and t-distributed stochastic neighbor embedding (t-SNE) were applied (35). These tools give an indication on the general (PCA) and the local structure, through minimizing the influence of large differences (t-SNE), of the data. Both PCA (Figure 1A) and t-SNE plot (Figure 1B) show a clustering of the biological replicates of each material, confirming the accuracy and reproducibility of gene expression profiling in this context.

The first principal component (PC1, x-axis in Figure 1A) encompasses the largest variations in the dataset (65.8% of the total variation) and reveals a cluster of two polymer/ceramic composite materials on the right of PC1 (cluster 1), whereas the majority of the materials (i.e. all ceramics, polymers, titanium, DBM and bECM) cluster together, having a more similar expression profile (in cluster 3), with respect to PC1 than to the profiles represented by cluster 1. The second principal component accounts for 6.5% of the total observed variation and is uncorrelated to the variation represented by PC1. This PC2 reveals the variations within the different classes of materials. MG-63 cultured on polystyrene and OCP coated titanium cluster separately (in cluster 4). The local structure of the data, reflected by the t-SNE plot, confirms that the ceramics generally cluster with each other and with a group containing the PLA, the PLA composite and bECM (cluster 3 in Figure 1B). In addition to the information obtained by PCA, t-SNE reveals that titanium, DBM, polystyrene, OCP coated titanium and TCP do not cluster with the other materials and BCP. Moreover, the different composites cluster separately in two distinct clusters (PCa,b in clusters 1 and PCc,d,e in cluster 2 in Figure 1B).

The aforementioned results indicate that the differential transcriptional profiles induced by the materials in cluster 1 in the PCA (polymeric composites PCa and PCb) and cluster 4 (OCP coated titanium and tissue culture polystyrene) mask the smaller differences between the different material types. In general, we observed that the gene expression differences are dominated by the material chemistry. However, it is not only chemistry that plays a role, since a general cluster is observed both in PCA and t-SNE that contains both ceramics and polymers. Indeed, it is reported that the biophysical properties of materials, such as surface structure and mechanical properties, largely influence cellular behavior (36-39).

Effects induced by surface treatment
PCA shows that the largest variation, represented along PC1, is significantly influenced by the difference between the composite materials treated with varying concentrations of NaOH (Figure 1A). The composite materials treated without NaOH (0 M NaOH; PCa) cluster with the ones treated with the lowest concentration (0.125 M; PCb) in cluster 1, in contrast to the composites treated with three higher concentrations (0.25, 0.5 and 1M; PCc-e), which group with the rest of the materials (cluster 3 in Figure 1A). This clustering suggests that the composite of PLA and nano-hydroxyapatite without any surface treatment or with only a mild surface treatment induces a specific but broad
Material-induced transcriptional profiles

Figure 1. Relationship between the genetic profiles of osteoblasts induced by the different materials. PCA plot representing the general transcriptional differences between the materials (A) and t-SNE plot focussed on the local differences between the samples without emphasis on the large variations (B).

Figure 2. SEM images showing the surface structure of the five polymeric composites treated with increasing concentrations of NaOH, PCa-e treated with 0 M, 0.125 M, 0.25 M, 0.5 M and 1 M respectively.
effect that is absent when the surface is etched with higher concentrations of NaOH. Differential expression between these two clusters of composites revealed 17173 probes (representing 11,148 detected genes) with a \( p \)-value below 0.05. As a reference, only 2145 probes are significantly differentially expressed between cells cultured on bECM and DBM, illustrating the massive changes occurring between these composite materials. SEM images (Figure 2) indicate a difference in surface roughness paralleling the alkali NaOH treatment; however no apparent distinction based on these surfaces can be made between the materials assembled in both clusters. Generic and qualitative chemical characterization of the surfaces shows the presence of both calcium and phosphate in all five materials, but no major differences in elemental composition (Fig. S1). However, conclusive observations on the differences between the surfaces require more precise and quantitative chemical and surface characterization methods. Previously, it was reported that this specific alkali treatment of similar composite materials did not alter the chemistry of the surface (40). It removes the polymer from the surface by hydrolysis and hence exposes uniformly nanostructured apatite particles. Increasing NaOH concentration increases the surface roughness, surface hydrophilicity and thickness of the apatite layer. It was shown that the increasing surface roughness of PLA composites by means of NaOH treatment resulted in decreased proliferation and increased ALP activity in hMSCs (40). Moreover, the gene expression profiles of MG-63 cultured on the uncoated or OCP-coated PLA with a lower molecular weight clustered with the ceramics and the PLA composites etched with higher concentrations of NaOH (Figure 1A). The composite from this low molecular weight PLA, PLAC, is fabricated similarly to the composites PCa-e and induces an expression profile that more closely resembles the composites with low NaOH treatment (cluster 1) than the uncoated PLA (in cluster 3). This observation is confirmed in the t-SNE plot where PLAC occupies cluster 1 together with the non- or mildly surface treated composites (Figure 1B).

To gain insights into the mechanisms behind the large differences induced by the composites in cluster 1, the top 500 probes contributing to the abovementioned PC1 were ranked based on their contribution weight and the differentially expressed genes with substantial effect size (absolute fold change > 4) were subjected to pathway analysis using IPA (Ingenuity Pathway analysis) and KEGG (Kyoto Encyclopedia of Genes and Genomes annotation database). The highly overrepresented signalling pathways in PC1 are “eIF2 signalling”, “regulation of eIF4 and P70S6K signalling” and “mTOR signalling” (Figure 3A). Noteworthy, the genes in these three overrepresented pathways largely overlap, as can be observed in the Venn diagram represented in Figure 3C. Also, the molecules contributing to the overrepresentation of “mTOR” and “eIF4 and p70S6K signalling” are nearly all involved in “eIF2 signalling” (41). The dominance of this pathway is mainly due to the negative contribution of 60 ribosomal protein-coding and four eIF genes in the first principal component. Eukaryotic initiation factor (eIF) is a family of proteins required for the initiation phase of protein translation (42). Mammalian target of rapamycin (mTOR) signalling regulates cell growth, proliferation motility, and protein synthesis and translation, reflecting the availability of growth factors, nutrients, ATP levels and amino acids. Downstream effects include translation of mRNA, ribosome biogenesis, and activation of transcription leading to mitochondrial metabolism (41, 43, 44). This is further supported when the overrepresented metabolic pathways are considered: “Oxidative phosphorylation”, “Glycolysis I” and “Gluconeogenesis” are overrepresented metabolic pathways in PC1 (Figure 3B), suggesting lower metabolic
activity and energy production. Then, to address the directionality of the observed differences, we analyzed the specific differences between the composites in clusters 1 and 2, reflecting the cellular responses modulated by NaOH treatment. Analyzing the pathways affected in the differentially regulated genes resulted in four significant signalling pathways, with minimum number of overlapping molecules. These pathways point again towards a lower transcriptional and metabolic state of the cells on the materials in cluster 1, similar to the PC1 analysis. Here, “RAN signalling” refers to protein and RNA transport into and from the nucleus, “mitotic roles in polo-like kinase” indicate a difference in cell cycle regulators and the “PI3K/AKT pathway” is important in signal transduction of cytokines, growth factors or extracellular proteins (Fig. S2A). Similarly, ribosome pathway, DNA replication, cell cycle and amino acid synthesis were enriched by these genes as evaluated by KEGG pathway analysis (45) (Fig. S2B).

Figure 3. Pathway analysis of the general differences between the materials. Significantly overrepresented signalling (A) and metabolic pathways (B) in the top 500 probes contributing to the first principal component of the PCA reflecting the largest variations between the different materials. The p-value indicates the probability of association of the molecules with the pathways and the ratio is calculated between the number of genes of interest associated with the pathway and the total number of genes that make up the pathway. The Venn diagram represents the overlap between the different genes represented in the three highly overrepresented signalling pathways (C).
adherens junctions” (actin cytoskeletal remodeling), “actin cytoskeleton remodeling” and “integrin signalling” (illustrated in Fig. S3) in both abovementioned analyses hint towards differential cell adhesion and cytoskeleton signalling. Notably, β1 integrin (ITGB1) is massively down regulated with a 14.9 fold change (average of three different microarray probes targeting ITGB1) in the two composites with no or low treatment in comparison to the three other composites. Also integrin alpha V is 7.15 fold down regulated. Besides, commonly assessed marker genes for osteogenic function (RUNX2, ALPL, BMP2 and COL1A1) did not reveal differences larger than 1.8 fold between the two clusters. Osteocalcin was three fold up- and osteonectin was six fold down regulated on the composites treated with higher concentrations of NaOH.

Using principal component analysis, we are able to compare the cellular responses to various materials. Based on the aforementioned transcriptional analyses, we noticed that the addition of nanoHA to PLA influences the surrounding cellular environment, thereby negatively affecting cell metabolism and downstream translation, which is prevented by the alkali treatment and removal of the polymer from the surface. Further analysis of these materials and their properties would unveil their influence on the large expression differences observed using genomic analyses.

Correlating gene expression to in vivo readouts

In vitro cellular behavior in response to a biomaterial may give useful insights in their observed in vivo responses. Correlating gene expression profiles to a known in vivo observation (e.g. coagulation or fibrous capsule formation upon prosthesis implantation, or peri-implant pseudotumor formation) may lead to new pathways to improve the biomaterial. Osteoinductivity, the in vivo bone inducing capacity upon implantation at heterotopic sites, is a very interesting material property for bone regeneration purposes. This phenomenon has been studied for decades in our group and by others, while a thorough understanding of the underlying molecular mechanisms is still missing. In order to unravel these mechanisms, we correlated in vitro gene expression patterns on various materials to their in vivo osteoinductive potential. We studied five ceramic materials with known osteoinductivity based on previously published data and observations from our group. Specifically, HAa, BCPa and TCPa are able to induce bone formation when implanted intramuscularly in a large animal model, whereas HAb and BCPb are not (38, 46-50). SEM images of the surface of these ceramics are depicted in Fig. S4. Interestingly, the remaining materials, not yet tested in vivo, can be compared in terms of genes differentially regulated in the materials with known osteoinductivity. It is important to note that the composite materials were removed as the large general differences might bias further analysis.

We first compared the osteoinductive (OI) ceramics (HAa, BCPa and TCPa) with the non-osteoinducative (nOI) ones (HAb and BCPb) resulting in a gene list of 2,576 probes significantly (p < 0.05) differentially expressed. Then, we used this list to plot all materials by PCA to compare them relative to the OI and nOI ceramics. The resulting plot revealed that the OI materials are spread in the upper region and the nOI cluster on the lower right part of the graph (Figure 4A). Most of the remaining materials cluster together with the nOI materials, including PLA, PLA coated with OCP and DBM. When considering
PC1, OI materials cluster in the middle, nOI are on the right and OCP coated titanium is observed together with MG-63 cultured on polystyrene on the left.

Next, we identified the genes that correlate quantitatively to the osteoinductivity, because the above discussed gene list appears to be highly influenced by the differential profile induced by BCPa (Figure 4A) as its induced profile clusters separately from the remaining OI materials which cluster towards the other materials. The quantity of osteoinduction refers to the amount of bone that is induced by the materials when implanted intramuscularly in diverse animal models. Indeed the three OI materials have gradually increasing bone formation, from HAA to BCPa to TCPa (38, 46, 50, 51). As such, genes

![Figure 4](image)

**Figure 4.** Analysis of the genes correlating to the osteoinductive property of ceramics. PCA plot based on genes differentially expressed (fold change > 1.4, p < 0.05) between the two non- (HAb and BCPb) and three osteoinductive materials (HAA, BCPa and TCPa) (A). PCA plot based on genes, with expression paralleling the extent of osteoinduction of the five materials (B).
were considered when their expression paralleled the extent of material-induced bone formation (i.e., increasing or decreasing expression from \([\text{HAb, BCPb}] < [\text{HAa}] < [\text{BCPa}] < [\text{TCPa}]\)) by applying a linear filtering constraint to the profile analysis. The resulting gene list contained 2,269 probes (correlation \(p < 0.05\)). The subsequent PCA (Figure 4B) for this list confirmed a difference between the ceramics, with the most osteoinductive ones (TCPa and BCPa) in the top and the non-osteoinductive ceramics on the right (BCPb and HAb). Furthermore, the remaining ceramics are distributed between the OI HAa and the nOI BCPb and HAb. The expression profile of MG-63 cultured on OCP coated titanium is shifted to the left and upwards, towards the expression of OI ceramics, in comparison to titanium. Indeed titanium coated with OCP has been reported to be osteoinductive when implanted intramuscularly, but it induces less bone in comparison to osteoinductive BCP after 6 weeks of implantation (52, 53). Moreover, as expected based on the effect of OCP coating, the expression profile induced by PLA shifted slightly towards the OI side in the PCA plot when an OCP coating is applied (Figure 4B). DBM, PLA and titanium are clustered near the nOI ceramics. In conclusion, using correlations to in vivo observations, PCA on diverse gene lists may describe the in vivo osteoinductive capacity of other materials.

The transcriptional profiles can give insight in the molecular mechanisms of in vivo biomaterial behavior. In order to impinge on the molecular responses in relation to osteoinductivity and quantity of bone formation, we further analyzed these two gene lists. To get a detailed insight in the differences between the two lists, we therefore de-convolved them separating the overlapping molecules and analyzed the subsets individually (Figure 5). First, IPA analysis on the molecules that are differentially expressed between OI and nOI, without the overlapping molecules from the quantitative OI list, pointed towards a role for cell adhesion and actin cytoskeleton remodeling (Figure 5). Indeed, all the listed pathways are based on focal adhesion molecules (\(\text{ITGB1}, \text{VCL}\)), cytoskeletal molecules (\(\text{ACTB}, \text{ACTG1}, \text{TUBB}\)) and PTEN. Notably, the overrepresentation of “VEGF signalling” pathway is also based on a few of these cytoskeletal molecules. Secondly, the genes correlating to the osteoinductive quantity (excluding the overlapping differentially expressed genes) revealed overrepresentation of signalling pathways such as “Wnt/B-catenin”, “regulation of epithelial-mesenchymal transition”, and “molecular mechanisms of cancer” (Figure 5). “Molecular mechanisms in cancer” describes the signalling pathways involved in intra- and intercellular communication leading to malignant tumor phenotypes. This broad pathway covers “GPCR signaling”, “ras/integrin signaling”, “AKT signaling”, “TGF-β/BMP signaling”, “WNT signaling”, “Notch and Hedgehog (Hh) signaling”, and “Death receptor signaling”. The molecules contributing to this overrepresentation are included in subsets of the other signalling pathways, mainly “BMP and TGF signalling” (e.g. BMP2, -4 and -6, BMPR2, TGF2), “WNT/bcatenin signalling” (e.g. several Frizzled and related genes, DKK1), “Interferon signalling”, and several MAP kinases and Rho family members. Thirdly, the overlapping molecules converge into “oncostatin M signalling”. Activated monocytes and lymphocytes produce oncostatin M (OSM), a member of the interleukin 6 (IL-6) hematopoietic cytokine family, acting locally on stromal cells, which in turn enhance their production of IL-6 and LIF to stimulate the inflammatory response. OSM has not only been shown to stimulate osteoblast mineralization and differentiation but also osteoblastic differentiation of MSCs (54). Moreover, OSM has been shown to enhance osteoinduction, when exogenously added to a critical sized defect (55).
The above analyses suggest that OI materials require oncostatin M signalling and differential cell adhesion, plausibly enabled by their differential microstructured surfaces (38, 48, 49) (SEM images in Fig. S4), and that the quantity of bone is determined by signalling pathways such as TGF and WNT (36, 56). To support this, we observed that the molecules involved in FAK signalling (Fig. S5) are mainly down regulated in OI materials.

### A

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### D

![Venn diagram illustrating overlapping molecules in both gene lists related to osteoinductivity (D). The significantly overrepresented pathways in the 114 genes solely differentially expressed between OI and nOI materials (A). The pathways significantly overrepresented in 1703 genes with expression correlating with the gradually increasing bone formation between the OI materials (B). The significantly overrepresented pathway in the 235 overlapping molecules between both gene lists (C). The p-value indicates the probability of association of the molecules with the pathways and the ratio is calculated between the number of genes of interest associated with the pathway and the total number of genes that make up the pathway.](image-url)
Focal adhesions (FA) link extracellular signals, integrating growth factor and integrin signaling from the ECM via the actin cytoskeleton to the nucleus (57). FA kinase (FAK) signalling controls cell adhesion and migration and together with downstream ERK/MAPK has been shown to be modulated by nano- and microtopographies, where more mature FA were observed on flat surfaces (58). FAK signalling and high FAK turnover has been associated with the disassembly of adhesion sites and subsequently increased migration, whereas reduced FAK signalling has been associated to a less migratory phenotype (59). Both PTEN and the Rho family members, together with the cytoskeletal genes regulate actin remodeling and cell shape, which might be a result of FAK signalling (60). Also, the analyses suggest that TGF-β and WNT signalling determine the amount of bone formation induced by ceramics. The link between molecules involved in TGF-β signalling, by means of BMP2 and -4 and BMPR2, WNT signalling and osteoblastic responses to rough titanium surfaces have been reported previously (36, 56). Overall, correlating in vitro gene expression profiles with in vivo observed effects gives insights in the molecular mechanisms induced by biomaterials, which may enable to unravel, in further in vitro and in vivo experimental validations, the mechanisms underlying material induced bone formation.

**Investigating gene expression in relation to material properties**

Correlating material properties to gene expression profiles improves the understanding and further development of materials. Here, chemistry is used as a readout to couple gene expression profiles to the presence of calcium and phosphate in the materials, since most materials studied in this dataset are ceramics or polymer-CaP composite materials. These ions (in solution or incorporated in a material) are known to greatly influence cellular responses, specifically for bone regeneration applications (25, 27, 46, 61-64). Surface characterization revealed that in addition to all ceramics and composites, bECM, OCP coated titanium and low molecular weight PLA contained calcium and phosphate, whereas DBM, titanium and uncoated PLA did not (Fig. S6).

Generally, the transcriptional differences could not be attributed to the presence of calcium and phosphate in the materials, since PCA on all genes did not reveal a cluster with calcium and phosphate baring materials (Ca/P; Figure 6A). Previous work on the biological effect of calcium phosphate ceramics has shown the up regulation of BMP2 in response to calcium (27, 65). As such, we postulated that calcium and phosphate containing materials differentially affect BMP2 signalling. To investigate this in the context of the biomaterials included in this study, three gene lists were produced. Firstly, 425 genes were listed (represented by 651 microarray probes) on evidence-based interactions with BMP2, by annotation, proven interaction, and pathway or functionally related, using the “endeavour” bioinformatics tool (33). Secondly, to obtain a list of probes that are selectively related to functional BMP2 signalling, the full microarray probe set was filtered to obtain 141 probes (representing 90 molecules) actively involved in TGF-β/BMP signalling based on information from the KEGG database (KEGG:04350). Lastly, this list of TGF-β/BMP signalling molecules was extended with related genes (using the endeavour bioinformatics tool). Principal component analyses and plots revealed separate clusters of Ca/P and non-Ca/P baring materials based on BMP2 related (Figure 6B) and TGF-β/BMP signalling molecules (Figure 6C). However, this clustering
was abolished when TGF-β/BMP signaling related molecules were included (Figure 6D). These findings indicate that the effect of calcium and phosphate incorporated in materials is specifically related to TGF-β/BMP signalling. Hence, the link between BMP2 and Ca/P via its specific signalling pathway described in the literature is validated by this dataset and the analyses.

Differential expression analysis between the calcium and phosphate containing materials and the others revealed 4,225 significantly different probes. While this is a large number of probes, their effect size is rather small. Only 22 genes showed differences higher than 2 fold (Table S1). Since all calcium and phosphate containing materials are combined,
it is possible that the comparison involves various materials with diverse properties that might average out the gene expression differences. To illustrate, the expression pattern of two genes, SLPI and ASPM, from the top five is represented in Figure 7 with an effect size of -3.2 and 2.7 fold. A clear trend between the calcium containing and calcium free materials can be observed. Specifically, the regulation of these genes is consistent over the different comparisons: between Ca/P coated titanium and uncoated, between bone matrix and demineralized bone matrix and between PLA coated with Ca/P and uncoated PLA (Figure 7).

Using the aforementioned analysis, we not only confirmed the previously known relationship between Ca/P and BMP2 in different materials, but we also hypothesize that the genes emerging from the differential expression analysis are regulated by calcium or phosphate.

### 4.4 Conclusion and outlook

This proof-of-concept study serves to illustrate the usefulness of a genomics approach in biomaterials research. Cell-biomaterial interactions are a subject of intense investigations and we propose transcriptomics as a tool with added value. Using defined analyses, correlations to material properties and readouts we discussed the effect of several materials and treatments on the biological response of cells.

General observations on gene expression profiles highlighted a dramatic effect of polymer/ceramic composite materials and alkali surface treatment, not observed in the expression of commonly used osteogenic markers, indicating the added value of genome wide screening. Moreover, we obtained more insights in the molecular mechanisms induced by osteoinductive materials by correlating transcriptional profiling to osteoinductivity, leading to defined hypotheses for further *in vitro* validations.
Material-induced transcriptional profiles

Specifically, we hypothesize that besides oncostatin M signalling, a down regulation of FAK signalling is required in consort with differential TGF-β and WNT signalling for materials to be osteoinductive. Additionally, by coupling expression profiles to material properties, we were able to confirm a previous observation reported in the literature, showing that that $BMP2$ and BMP signalling are influenced by calcium and phosphate.

From this study, new hypotheses evolved from the observed and analysed differential cell behaviour induced by materials, which can be further validated in vitro. Together with future applications, this approach will aid researchers in understanding cellular responses in relation to material properties, which will promote the development of more effective biomaterials for applications in regenerative medicine.
4.5 References


Material-induced transcriptional profiles


[65] Chai YC, Roberts SJ, Schrooten J, Luyten FP. Probing the Osteoinductive Effect of Calcium Phosphate by Using an In Vitro Biomimetic Model. Tissue Engineering Part A 2011;0(0):null.
Supplementary Information

4.6 Materials and methods

Synthesis of calcium phosphate ceramics as obtained from Xpand Biotechnology
Five different calcium phosphate (CaP) ceramic particles were provided by Xpand Biotechnology BV (Bilthoven, The Netherlands). Hydroxyapatite powder (with a Ca/P ratio of 1.67) was purchased from Merck, calcium deficient apatite powder with a Ca/P ratio of 1.64 was wet precipitated with calcium nitrate and di-ammonium hydrogen phosphate [1], calcium deficient apatite powder with Ca/P ratio of 1.50 was wet-synthesized with calcium hydrogen oxide. Porous green ceramic bodies were prepared with diluted H₂O₂ solution and wax particles at 60°C. CaP ceramics were then obtained by burning out wax and sintering at high temperatures for 8 hours. HAa and HAb were obtained from green bodies made from hydroxyapatite powder at 1150 °C and 1250 °C; BCPa and BCPb were obtained from green bodies made from calcium deficient apatite powder with a Ca/P ratio of 1.64 at 1150 °C and 1300 °C respectively; TCP ceramic was finally obtained from the green bodies made from apatite powder with a Ca/P ratio of 1.50. Ceramic particles with the size of 1 - 2mm were subsequently fabricated and prior to further usage, were ultrasonically cleaned with acetone, 70% ethanol and demineralized water, then further dried at 80 °C, and sterilized by autoclavation.

Synthesis of calcium phosphate ceramics as obtained from CAM Bioceramics
Five different ceramics were provided by Cam Biomaterials B.V. (Leiden, The Netherlands). Hydroxyapatite (> 99 %) and β-TCP (> 99 %) were both sintered at 1100 °C (HAc and TCPb). Three biphasic calcium phosphates were sintered at 1150 °C; HA/TCP (78/22), HA/TCP (70/30) and HA/TCP (61/39). All the materials were sieved to obtain particles in the range of one to two millimetres.

Synthesis composite materials as provided by Xpand biotechnology
The precipitated apatite powder was extruded with poly (D,L–lactide) (declared inherent viscosity 4.22 dL g–1; HuiZhou, P.R. China) to prepare a dense 50/50 wt.% nano-sized HA/polymer composite. Nano-sized HA powder was prepared through a wet-precipitation reaction as described elsewhere (66). The extrusion was done using a mini vertical conic twin–screws extruder (RD11– H –1009–025–4, DSM Research BV, Geleen, the Netherlands; top screw diameter: 10 mm; bottom screw diameter: 4.15 mm; screw thread: 8 mm; screw length: 108 mm) with screw speed set at 100 ± 1 rpm for a mixing time of 6 ± 0.5 minutes. The extrusion temperature was set at 205 ± 1 °C to allow enough polymer softening with minimal thermal degradation. The samples were then surface etched in sodium hydroxide (NaOH, Merck, Darmstadt, Germany) with different concentrations (i.e. 0 M, 0.125 M, 0.25 M, 0.5 M, 1 M) at room temperature for 5 ± 0.5 minutes to obtain five composite materials with different surface roughness levels (i.e. PCa, PCb, PCc, PCd, PCe respectively). By grinding the extruded composite bodies at 14,000 rpm with a centrifugal mill (ZM 100, Retsch GmbH, Haan, Germany) and sieving, granules with a fraction size of 0.5 - 1 mm were obtained. All the samples were then sterilized with ethylene oxide (IsoTron Nederland BV, Ede, the Netherlands) and used for experimental work.
Synthesis composite materials
Composite materials were produced by mixing 50 wt.% nano-sized HA with 50 wt.% amorphous poly (D,L-lactic acid) (PLA), with a molecular weight of 59,000 Da (Purac), as published previously (67). In short, nano-sized HA powder was prepared as described above and the composite was produced by extrusion using a twin screw extruder with conical non-converging screws (Artecs BV, Enschede, the Netherlands) (150 °C and 100 rpm screw rotation). PLA samples were prepared using the same process. Following extrusion, PLA and the PLA composite (PL-C) rods were grinded and sieved into particles of 0.5 - 1 mm.

Titanium
Porous titanium rods were produced from Ti6Al4V using selective laser melting (Layerwise, Leuven, Belgium) with 5 mm diameter and 10 mm length, as reported previously (68). These scaffolds were designed using a dodecahedron unit cell as a template structure with 120 µm struts. All samples underwent postproduction chemical and heat treatment to increase surface roughness. Briefly, the titanium rods were immersed in 5 M NaOH solution at 60 °C for 7 h, washed in water for 12 h at room temperature, followed by immersion in 5% HCl for 60 °C for 5 h and again washed in water for 24 h at room temperature. Lastly, the titanium scaffolds were heated to 600 °C at a rate of 5 °C/min in an electric furnace in ambient air pressure, holding the temperature at 600 °C for 1 h.

OCP coating
The PLA composite particles and Titanium rods were then coated using a two-step biomimetic method. In the first step, the samples were immersed in a simulated body fluid (SBF) at 37 °C in order to coat the surface with an amorphous CaP phase. The crystalline CaP phase was deposited on the pre-treated surfaces by immersing the particles in a Calcium phosphate saturated solution at 37 °C.

bECM and DBM provided by EMCM B.V.
Donor-derived femoral heads were used to prepare decellularized bone (bone extracellular matrix; bECM) and demineralized bone matrices (DBM). Trabecular bone was serologically and bacteriologically tested and was free of micro-organisms. Subsequently, the bone pieces were treated with supercritical CO₂, H₂O₂, NaOH and NaH₂PO₄. Finally, bone pieces were treated with 96% and 100% ethanol and were vacuum dried. Both bECM and DBM samples were shaped into particles of 1 to 2 mm, and DBM samples received an additional 0.5 M HCl treatment. Prior to use, samples were gamma sterilized.
4.7 References


Chapter 5

Distinct transcriptional profiles controlled by chemistry and surface topography in calcium phosphate ceramics
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Distinct transcriptional profiles controlled by chemistry and surface topography in calcium phosphate ceramics

Nathalie Groen, Yuan Huipin, Gülistan Koçer, Faustin Mbuyi, Pamela Habibovic, Roman Truckenmüller, Clemens A. van Blitterswijk and Jan de Boer

Abstract

The molecular mechanisms and the responsible material properties underlying material-induced bone formation remain largely unknown. Here we used a transcriptomics approach to investigate the cellular and molecular mechanisms induced by various calcium phosphate ceramics. Transcriptional profiling in different cell types allowed us to identify 13 genes correlating to the in vivo osteoinductive capacity and concomitant microstructural properties of materials. The expression of these genes was further verified using cells cultured on osteoinductive tricalcium phosphate ceramic discs with tailored microstructural properties. To determine the effect of individual material properties on the expression of the identified genes, we de-convoluted the surface structure and chemical composition. We used microfabrication protocols to replicate the surface of non- and osteoinductive ceramics into polystyrene films and were able to attribute the expression of TNC and HAS2 to surface structure effects, whereas KIAA1199, MGP and TNC were affected by extracellular calcium and phosphate. This study points towards the important role of the extracellular matrix (ECM) generated by the cells in response to properties presented by osteoinductive materials. We propose a mechanism by which the surface structure and ceramic-mediated ion release induce the expression HAS2 and TNC and the repression of KIAA1199, thereby modulating the molecular weight of the produced hyaluronic acid secreted in the ECM. Moreover, we correlated the up regulation of MGP and TNC to the microstructural properties of the materials, suggesting their presence in the ECM of cells cultured on osteoinductive ceramics.
5.1 Introduction

In the past decades, several classes of biomaterials have shown the ability to induce bone formation upon implantation in a non-bony environment (osteoinductivity) and promote bone ingrowth at orthotopic sites (osteocomductivity). Calcium phosphate (CaP) ceramics, being one of these classes, possess bone-resembling mineral phases and are not only biocompatible and osteoconductive, but are interestingly in some specific cases also osteoinductive. This special type of ceramics has been used and studied within the context of repair of large bone defects for these reasons. Developments in the field have provided us with a wide variety of natural and synthetic CaP based materials that can be produced with tailored material properties for dental and orthopaedic applications.

The importance of the different material properties for the intrinsic osteoinductive behaviour of these materials both in vitro and in vivo have been extensively studied and reviewed [1-3]. Briefly, macroporosity allows blood vessel ingrowth and creates a protected environment for the new bone to form. Moreover, the chemistry (molecular composition and stoichiometry), physicochemical properties (crystallinity, surface energy, solubility and surface charge), surface topography (grain size and roughness) and microporosity (pore volume below 10 µm) of the ceramics influence the dissolution/precipitation kinetics and protein adsorption. More specifically, the dissolution of calcium and phosphate and the surface microstructure have an important effect on osteogenic cell response. Calcium released from the ceramics affects osteoblastic differentiation, which is proposed to occur via the CaMK2α/CAM, CREB, PKC and ERK1/2 pathway [4-6]. Similarly, phosphate, besides being a component of mineralized extracellular matrix (ECM), is able to actively induce matrix mineralization and its osteogenic effect has been attributed to altered levels of ERK1/2 [6-9]. In parallel, the surface microstructure, defined as the combination of the grains and pores, has been shown to influence the existence and the extent of osteoinductive capacity [10-12]. Beyond ceramics, the surface roughness, topography or structure of substrates have been extensively studied in the scope of stem cell behaviour and differentiation [13-15]. The differential cellular response induced by variations in microstructure is suggested to be mediated via differential protein adsorption, integrin binding, focal adhesion dynamics, actin cytoskeleton remodelling and downstream activation of MAPK and ERK1/2 [16-23]. Moreover, the surface microstructure of ceramics influences the specific surface area and hence has a direct impact on the dissolution properties, underlining the complex nature of these material systems with intertwined properties and effects.

Although a great deal of research has been conducted on the tailorability of the physicochemical and structural properties of ceramics to optimize in vivo bone formation, the mechanisms underlying material-induced osteoinductive behavior and knowledge of the responsible material properties remain largely undefined. Rapid developments in the field are hampered by inadequate in vitro models and the necessity of in vivo assessment [24]. To tackle this, gene expression profiling could be of use, since it allows a global measurement of gene expression status assessing thousands of probe sets simultaneously representing thereby nearly all known protein-coding genes. Historically, gene expression profiling has been applied to unveil biological signalling pathways, study mechanisms involved in development or to study stem cell niches. Exemplary are the studies on hematopoietic stem cells and their progeny or immune cells and their function [25-
Disease states and cancer types have also been identified with respect to normal tissues [29, 30]. Furthermore, genomics is commonly used in the pharmacological field to investigate the effect of therapeutics and small molecules [31].

Within the field of tissue engineering and regenerative medicine, gene expression profiling has been successfully used to identify pathways and molecules involved in the early response of articular cartilage to injury in an in vitro model, which was confirmed in osteoarthritic cartilage [32]. Human mesenchymal stem cells (MSCs) have also been profiled in vitro to identify genes predictive for in vivo bone formation by implanted MSCs [33, 34]. Gene networks have been identified in human periosteal derived cells cultured in vitro and in vivo on a composite of ceramics/collagen aiming at improving in vivo bone formation by in vitro pre-treatment of the cells [35]. The effects of various surface structures on cellular responses were investigated by comparing the transcriptional variations [20, 36-38]. Gene expression based analysis has been performed on bone marrow derived MSCs cultured on electrospin and spin coated materials and mineralizing MSCs to compare the mechanisms induced by the substrate chemistry and structure with the biochemical stimuli [39]. Moreover, we previously reported the differentially regulated genes in response to two ceramic materials with different osteoinductive and material properties [5].

In order to study the cellular effects induced by ceramic properties in the context of osteoinductivity, we used transcriptional profiling on a set of ceramics with varying properties. To further unravel the observed cellular response, we de-convoluted the ceramic properties into the effect of chemical composition and the effect of the surface structure. Thereafter, we mimicked the surface architecture of ceramics in polystyrene films and modelled the calcium and phosphate release to validate the identified gene expression responses.

5.2 Materials and methods

Materials

Synthesis of calcium phosphate ceramics

In this study, multiple ceramic materials are used as macroporous particles or as “dense” discs without macro pores or as “dense” cylinders with cut gaps. Firstly, five different calcium phosphate (CaP) ceramic particles ranging from one to two millimeter were prepared from apatite powders with Ca/P ratio of 1.67 (HA), Ca/P ratio of 1.64 (BCP) and CaP ratio of 1.50 (TCP). Briefly the apatite powders were either purchased (for HA) and wet-synthesized with calcium and phosphate solutions (for BCP and TCP), then green bodies were made with diluted H2O2 solution (1-2%) and wax particles at 60 °C, subsequently the green bodies were obtained after sintered at high temperatures for 8 hours. HA ceramics were sintered at 1150 °C (HAi) and 1250 °C (HAni), BCP at 1150 °C (BCPi) and 1300 °C (BCPni), β-TCP at 1050 °C (TCPp) respectively. Prior to further usage, these particles were ultrasonically cleaned with acetone, 70% ethanol and demineralized water, then further dried at 80 °C, and sterilized in an autoclave.

Secondly, “dense” green ceramic bodies were prepared from apatite powder with a Ca/P ratio of 1.50 using diluted H2O2 solution (0.1%) at room temperature and sintered at 1150
°C (TCPa), 1050 °C (TCPb) and 1000 °C (TCPc) respectively. “Dense” ceramic discs (Ø9 x 1 mm) and “dense” ceramic cylinders (Ø9 x 10 mm) with two cut gaps (0.8mm in width) were machined from the ceramic bodies using a lathe and a diamond-coated saw microtome (Leica SP1600; Leica, Solms, Germany) and were subsequently ultrasonically cleaned, and heat sterilised at 160 °C for 2 h.

**Material characterization of the calcium phosphate ceramics**

Chemical properties and crystal structures of CaP ceramics were analysed using Fourier transform infrared spectroscopy (FTIR; Spectrum100, Perkin Elmer Analytical Instruments, Norwalk, CT, USA) and X-ray diffraction (XRD; Miniflex, Rigaku, Japan).

The macro- and microstructure of the different ceramics was evaluated using a SEM (XL30, Environmental SEM-Field Emission Gun, Philips) in the secondary electron mode. The grain sizes of the ceramics were determined by measuring the diameter of 60 different grains from three independent SEM images using Image J [40].

In addition to SEM analysis, the pore size distribution, the microporosity (volume percentage of pores smaller than 10 µm) and specific surface area were determined for the different ceramics using mercury intrusion porosimetry (Quantachrome Instruments, Pore Master, USA). Density measurements were performed to determine the true (skeletal) density of the materials using a helium pyconometer (Accu Pyc II 1340 Gas pyconometer, Micrometrics). Then, porosimetry data were analysed with Pore Master software using mass, density and intruded volume as input parameters.

Calcium and phosphate ion release profiles were determined in simulated physiological saline (SPS; 137 mM Na⁺, 177 mM Cl⁻ and 50 mM HEPES, pH 7.3 at 37 °C in demineralized water) at various time points for the different materials studied. Seventy-five µL of CaP ceramic granules were kept in 1 mL SPS solution at 37 °C (mildly shaking) for the indicated time points, without refreshing the SPS solution. The calcium content, hence the release from the ceramics, was determined using a QuantiChrom Calcium assay kit (BioAssay Systems). The release of phosphate into SPS was analysed using a phosphate kit (Bioquest Inc, CA, USA).

In vivo characterization of the bone forming capacity of the studied ceramics

Surgery was performed on six adult dogs (mongrel, male, 10-15 kg, 1-2 year old) after obtaining the permission of the local animal care committee (Animal Centre, Sichuan University, Chengdu, China). All surgeries were conducted under general anaesthesia
by abdominal injection of sodium pentobarbital (30 mg/kg body weight). After shaving and sterilizing with iodine, a longitudinal skin incision and a median fascial incision were made, and the paraspinal muscles were exposed. Muscle pouches with the distance larger than 2 cm were made along the paraspinal muscle with blunt separation. One-milliliter ceramic particles of HA/ni, HA/i, BCP/ni, BCP/i and TCP/p, one “dense” cylinders with cut gaps of TCP/a, TCP/b and TCP/c were loaded in individual muscle pouches. After the implants were sealed in the muscle pouches with silk suture, the skin was sutured as well with silk suture. Following the surgeries, the animals were intramuscularly given buprenorphine (0.1 mg per animal) for two days to relieve pain and penicillin (40 mg/kg) for three days to prevent infection. After the surgical operation, the animals were allowed full weight-bearing and received normal diet.

The dogs were sacrificed after 12 weeks with an abdominal injection of an excessive amount of sodium pentobarbital. Implants were harvested with surrounding tissues, trimmed, fixed in 4% formaldehyde, dehydrated with a gradient ethanol scale and embedded in PMMA. Non-decalcified sections were made using a diamond saw (SP-1600, Leica, Germany), stained with 1% methylene blue (Sigma-Aldrich) and 0.3% basic fuchsin (Sigma-Aldrich) solutions for light microscopic observations.

**Imprinting of surface topography of ceramics in polystyrene**

In order to replicate the full morphological structure of ceramics on polystyrene substrates, the four TCP ceramic discs were imprinted in an intermediate mould of cyclic olefin copolymer (COC) (T_g=140 °C, 100 µm thickness). A hot embossing nano-imprint lithography (NIL) machine (NIL 6°, Obducat, Malmö, Sweden) was used, and imprinting was performed at 160 °C and 50 bars pressure for 300 s, followed by cooling and demoulding at 60 °C. Thereafter, the intermediate mould was incubated with 1M HCl for 1 h in order to remove CaP layer from the COC polymer without influencing the topographical features. The COC templates were treated with oxygen plasma (50 sccm O_2, 50 W, 75 mTorr, 30 s) prior the coating with fluoroctatrichlorosilane (FOTS, Sigma). Finally, the intermediate mould was used to replicate the desired features in polystyrene (PS, oriented PS, Polyflex clear grade 75 µm thickness, Sidaplax) thin films. Again, hot embossing was used at 115 °C and 50 bar pressure for 300 s, followed by cooling and demoulding at 60 °C. After imprinting, the polystyrene replicas were treated with oxygen plasma and sterilized using 70% ethanol. Characterization of the replication was done by visual observation following SEM imaging.

**Cell culture**

The osteosarcoma cell line MG-63 was expanded in medium consisting of α-minimal essential medium (α-MEM, Life Technologies) supplemented 10% fetal bovine serum, 0.2 mM ascorbic acid (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin and 100 µg/ml streptomycin. Medium was refreshed twice a week and further experiments were performed at 37 °C and 5% CO_2.

To investigate the effect of the materials on MG-63, 200,000 cells resuspended in 100 µL medium were seeded per 150 µL of particles pre-wetted with medium and allowed to attach for 4 h before adding additional medium. The cell-material constructs were
cultured in their respective medium for 48 h or 7 days. As a reference, MG-63 were seeded at low density (5 000 cells per cm²) and at high density (25 000 cells per cm²) on tissue culture polystyrene and cultured for the mentioned time periods.

To address the effect of calcium and phosphate on the expression of the genes of interest, inorganic phosphate was added as a mixture of NaH₂PO₄ and Na₂HPO₄ (pH 7.2, Sigma-Aldrich) to a final concentration of 10 mM in basic culture medium (containing 1 mM Pi and 1.8 mM Ca). Calcium was added as a solution of CaCl₂ (Sigma-Aldrich) to a final concentration of 7.8 mM. MG-63 were seeded on tissue culture polystyrene (Nunc). After obtaining a confluent layer, the cells were exposed to elevated levels of calcium and/or phosphate 48 h and 7 days. To investigate the effect of the superficial microstructure of the ceramics on the expression of the genes of interest, MG-63 were seeded onto the replicated polystyrene surfaces in basic medium at a 5000 cells per cm² and cultured for 7 days.

**Transcriptional profiling**

**RNA isolation**
After cell culture on the different materials, total RNA was isolated using the Nucleospin RNA isolation kit (Macherey-Nagel). Then, from 275 ng of RNA, cRNA was synthesized using the Illumina TotalPrep RNA amplification Kit, according to the manufacturer’s protocol and both RNA and cRNA quality were verified on a Bioanalyzer 2100 (Agilent).

**Gene expression profiling**
Microarrays were performed using Illumina HT-12 v4 expression Beadchips. Briefly, 750 ng of cRNA was hybridized on the array overnight, after which the array was washed and blocked. Then, by addition of streptavidin Cy-3, a fluorescent signal was developed. Arrays were scanned on an Illumina Beadarray reader and raw intensity values were background corrected in BeadStudio (Illumina). Further data processing and statistical testing were performed using R and Bioconductor statistical software [41]. Graphical visualization of the data was obtained using GeneSpring software (Agilent Technologies Inc. and Strand Life Sciences Pvt. Ltd.). The probe-level raw intensity values were quantile normalized and transformed using variance stabilization (VSN). A linear modeling approach with empirical Bayesian methods, as implemented in Limma package [42], was applied for differential expression analysis of the resulting probe-level expression values. *P*-values were corrected for multiple testing using the Benjamini and Hochberg method [43]. Genes were considered differentially expressed when a corrected *p*-value below 0.05 was reached.

**Validation datasets**
The genes of interest (GOI) from the above experiment were independently validated in three other datasets. Firstly, a dataset published by Barradas *et al.* [5] was used. Specifically, donor-derived human mesenchymal stem cells (hMSC) were cultured on HAni and TCPp (the same materials as in this study) in medium (same composition as described above) supplemented with 10 nM dexamethasone for various time points including 48 h.
The second and third dataset used for validation of the identified genes included the same HAni and TCPp ceramic particles on which an immortalized donor derived human mesenchymal stem cell line (iMSC) and MG-63 were cultured (in medium with the same composition) for 48 h, as published previously [44].

Quantitative polymerase chain reaction
In order to investigate the regulation of gene expression in response to CaP ceramics, cDNA was prepared from RNA according to the manufacturer’s instructions in a 20 µL total volume (BioRad, USA). Quantitative RT-PCR was performed in a 20 µL volume with 0.01 nmol of forward and reverse primers. Reaction conditions were adjusted with annealing at 60 °C for 15 seconds and extension at 72 °C for 15 seconds for 35 cycles. β-2-microglobulin (β2M) was used as a house-keeping gene and relative expression were determined using the ΔΔCT method. Primer sequences are given in Table S1.

Statistics
All experiments were performed in triplicate and are presented as the mean ± standard deviation. Assays were analyzed using one or two way ANOVA with Bonferroni’s multiple comparison test comparing the groups of interest. Statistics used during bioinformatics characterization are explained in detail in the respective subsection on gene expression profiling.

5.3 RESULTS
Ceramics with varying properties and distinct osteoinductive capacities
The particular interest in calcium phosphate ceramics arises from their similarity to the mineral phase of bone. These materials present suitable alternatives to autologous bone implants because of their intrinsic property to promote and, in specific cases, induce the formation of bone. Besides the ion release profiles, the surface microstructure of these ceramics has been suggested to play a key role in their bioactive and osteoinductive capacity. To better understand the biological role of the surface structure, we compared ceramic materials with different chemical compositions and microstructural properties.

Figure 1. SEM photographs depicting the microstructure of five studied calcium phosphate ceramics. The upper images represent the non-osteoinductive hydroxyapatite (HAni) and biphasic calcium phosphate (BCPni). The lower images represent the osteoinductive HAi, BCPi and tricalcium phosphate particles (TCPp).
Therefore, three different chemistries were studied: hydroxyapatite (HA), \( \beta \)-tricalcium phosphate (TCP) and a mixture of 80 ± 5% HA and 20 ± 5% TCP referred to as biphasic calcium phosphate (BCP). Interestingly, by altering the process parameters during production, e.g. sintering temperature, variations in structural properties were obtained (represented by SEM images in Figure 1). As such, for both HA and BCP chemistry, one material with a lower grain size, higher microporosity, and higher specific surface area (HAi and BCPi, referred to as microstructured) was obtained in comparison to their counterparts (HAni and BCPni, referred to as unstructured) (Figure S1). The TCP particles were sintered at 1050 °C (TCPp) resulting in structural properties similar to HAi and BCPi (Figure 1 and S1). According to mercury intrusion porosimetry, HAni and BCPni did not show the presence of pores (Figure S1). Although HAi and TCPp have similar sized pores (approximately 0.8 µm), TCP particles have a higher incremental pore volume reflecting higher porosity. Similarly, more pores are present in BCPi but with a slightly larger mean pore diameter (1 µm).

Intramuscular implantation of these materials revealed a strong correlation between the structure of the materials and bone formation (Figure 2). HAni fails to induce bone formation when implanted ectopically in a dog model, whereas BCPni forms minimal amounts of bone in only 2 out of 6 samples. The osteoinductive materials HAi, BCPi and TCPp form bone in 6 out of the 6 animals tested with HAi inducing small amounts of bone in comparison to BCPi and TCPp.

Chemical and compositional analyses confirmed that the material pairs different in microstructure (BCPni - BCPi and HAni - HAi, respectively) had the same chemistry. While TCP ceramics possessed less than 5 wt.% HA. HA ceramics were pure in composition (Figure S2). The osteoinductivity of biomaterials has been suggested, among other hypotheses, to be mediated by protein adsorption [1, 2]. To this end, we quantified the adsorbed proteins after immersion in fetal bovine serum containing medium. Interestingly, the materials with unstructured surfaces (HAni and BCPni) adsorbed more protein (corrected for surface area) after 2 h and 24 h incubation than their microstructured equivalents and TCPp (Figure S3). Similarly, a strong correlation between osteoinductivity and calcium and phosphate release has been reported previously [5, 12, 45]. We observed more calcium release over a period of two weeks, which is consistent with the increased porosity and surface area of the microstructured materials.
from the TCPp, the most osteoinductive ceramic, in simulated physiological saline (SPS) than both types of HA and BCP materials (Figure S3). Although both BCP materials had a similar calcium release, the release from HAI was comparatively higher, and the release from HAni was the lowest among all materials. Furthermore, no phosphate release was detected from HAni over the tested time frame, whereas the release from HAI gradually increased. In contrast, the initial phosphate dissolution of the two BCPs was more pronounced, with the BCPi higher at the early time points (24 and 48 h) to ultimately converge to the level of HAI around 100 µM. TCPp showed a similar burst release but was followed by a steady and higher dissolution over time than the other materials. Indeed, TCPp is known to dissolve faster compared to BCP and even more compared to HA [46].

Overall, we produced five ceramic materials with distinct microstructural surface features with concomitant bioactive characteristics. These well-defined materials enable us to study the effect of surface microstructures on the molecular mechanisms underlying cell response.

Molecular responses correlating to osteoinductive capacity and microstructure property

To understand the biological effect of these ceramics and their specific properties, we investigated the differences at the gene expression level. Filtering the overlapping genes that were consistently differentially expressed in different cell types and independent experiments increases the biological confidence while reducing the false positives. Thus,

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Comparison based on osteoinductivity</th>
<th>Significance</th>
<th>Overlapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-63</td>
<td>HAni - HAI</td>
<td>p &lt; 0.05</td>
<td>13 genes</td>
</tr>
<tr>
<td></td>
<td>BCPni - BCPi</td>
<td>p &lt; 0.05</td>
<td>HAS2, TPM1,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>185 probes</td>
<td>ATF3, CEBPD,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EGLN1, ELL2,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RBPJK, KIAA1199,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MKNK2, PPP1R3B,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MGP, TNC, TNFRSF21,</td>
</tr>
<tr>
<td>iMSCs</td>
<td>HAni - TCPp</td>
<td>Top 500</td>
<td></td>
</tr>
<tr>
<td>MG-63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hMSCs (+-dex)</td>
<td></td>
<td></td>
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</tbody>
</table>

*Figure 3. Gene expression profiling.* Genes of interest (GOI) were identified in correlation to the osteoinductive property in three different cell types and independent microarray experiments. The differentially expressed genes between non- and osteoinductive ceramic materials were compared in MG-63 (two independent microarray datasets), iMSCs and hMSCs. This comparison resulted in 13 overlapping genes, listed on the right, which were further studied.
we identified genes of interest (GOI) that correlate with the osteoinductive property in three independent DNA microarray datasets with three cell types cultured on microstructured and unstructured ceramic materials (Figure 3). First, we compared the gene expression profiles of osteoblasts (MG-63) cultured for 48 h on HAni with HAi and on BCPni with BCPi separately. In order to focus on the differences specifically induced by the microstructural properties of the ceramics, we considered that making comparisons within each material chemistry would reduce the effects related to chemistry. The first comparison using HA-based materials revealed 185 probes, targeting 132 genes, significantly differentially expressed, whereas 3765 probes, targeting 2919 genes, were differentially expressed for the two BCP-based materials. From these two gene lists, 150 probes, targeting 115 genes, overlapped. Then, a dataset based on human mesenchymal stem cells (MSCs) cultured for 48 h on HAni and TCPp (a comparison between osteoinductivity and chemistry) in dexamethasone containing culture medium, as described by Barradas et al. [5] was used to narrow down the gene list. Secondly, a similar DNA microarray dataset including immortalized MSCs (iMSCs) and osteoblasts (MG-63) cultured for 48 h on HAni and TCPp was included [44]. From the 150 overlapping probes differentially expressed in MG-63 based on the four ceramics, 13 genes (summarized in Table 1) were also differentially expressed in iMSCs or human MSCs and MG-63 cultured on two materials, HAni and TCPp, with a difference in microstructural properties such as pore and grain size, microporosity, chemical composition and osteoinductivity.

Next, we confirmed the correlation of these 13 identified genes to microstructural properties in an independent experiment using quantitative RT-PCR to assess the gene expression in MG-63 cultured on the five ceramics. In Figure 4, the expression profiles of the validated GOI on the different materials are represented. Note that only the expression on the particles is considered in this part, depicted in light blue in the graphs. From the identified 13 genes, the expression of 12 genes could be assessed from

<table>
<thead>
<tr>
<th>Official Symbol</th>
<th>Name</th>
</tr>
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<tbody>
<tr>
<td>HAS2</td>
<td>Hyaluronan synthase 2</td>
</tr>
<tr>
<td>TPM1</td>
<td>Tropomyosin 1 (alpha)</td>
</tr>
<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
</tr>
<tr>
<td>CEBPD</td>
<td>CCAAT/enhancer binding protein (C/EBP), delta</td>
</tr>
<tr>
<td>EGLN1</td>
<td>Egl-9 family hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>ELL2</td>
<td>Elongation factor, RNA polymerase II, 2</td>
</tr>
<tr>
<td>KIAA1199</td>
<td>Cell migration inducing protein, hyaluronan binding</td>
</tr>
<tr>
<td>MKNK2</td>
<td>MAP kinase interacting serine/threonine kinase 2</td>
</tr>
<tr>
<td>PPP1R3B</td>
<td>Protein phosphatase 1, regulatory subunit 3B</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix gla protein</td>
</tr>
<tr>
<td>TNC</td>
<td>Tenascin C</td>
</tr>
<tr>
<td>TNFRSF21</td>
<td>Tumor necrosis factor receptor superfamily, member 21</td>
</tr>
<tr>
<td>RBPJ</td>
<td>Recombination signal binding protein for immunoglobulin kappa J region</td>
</tr>
</tbody>
</table>

Table 1. Overview of the 13 genes of interest identified in correlation to the osteoinductive and microstructural property of calcium phosphate ceramics.
which eight genes showed a consistent expression pattern over the microstructured and unstructured ceramics. HAS2, KIAA1199, ATF3, TNFRSF21 and TNC were consistently differentially regulated between the unstructured and the microstructured ceramics within each chemistry (i.e. concordant up- or down regulated between HAni and HAI and between BCPni and BCPi in the microarray experiment). There was no apparent difference between the unstructured ceramics and tissue culture polystyrene. HAS2, ATF3, MKNK2 and MGP were significantly differentially expressed between the MG-63 cultured TCP particles compared to the unstructured HAni and BCPni. In comparison to MG-63 cultured on “flat” tissue culture polystyrene, the expression of HAS2, KIAA1199, ATF3, MGP, TNFRSF21, CEBPD and MKNK2 differed consistently.

Figure 4. Gene expression validation. Relative mRNA levels of the identified GOI on non- and osteoinductive HA and BCP, osteoinductive TCP, and on three osteoinductive TCP discs (a, b and c) with a variation in surface structure. The expression of HAS2 (A), KIAA1199 (B), CEBPD (C), ATF3 (D), MKNK2 (E), TNFRSF21 (F), TNC (G), MGP (H) and BMP2 (I) after 48 h of culture on the different ceramics is altered. ANOVA with Bonferroni correction was used to test the statistical significance of the expression between the non- and osteoinductive ceramics (∗p < 0.05, **p < 0.01, ***p < 0.001). ANOVA with Dunnett correction was used to test the statistical significance of the differential expression between the conditions and MG-63 cultured on tissue culture polystyrene(#p < 0.05, ##p < 0.01, ###p < 0.001).
on the microstructured particles, in agreement with the microarray data. Although the remaining GOI mostly showed a correlation to microstructure, their effect size was too low for statistical significance and biological relevance (data not shown). Cell density has been shown to influence cell behaviour and gene expression, however, most of the GOI did not reveal to be differentially regulated when the cells were grown at low or high density on tissue culture polystyrene (grey bars in the expression graphs, Figure 4). Only ATF3 was significantly 2.5 fold down regulated and HAS2 expression appeared to be decreased in high density compared to low density culture. Additionally, we assessed the expression of BMP2, as its relation to osteoinduction and its responsiveness to calcium and phosphate has been shown previously, but was not detected in our microarray comparison [4-6]. Consistent with these observations, BMP2 was significantly up regulated on the TCP particles compared to the unstructured HAni, BCPni and polystyrene (Figure 4I).

After seven days of culture, the expression of TNC and KIAA1199 showed a persistent pattern (Figure S4). Strikingly, KIAA1199 was highly down regulated, between 20 and 40 fold, on the microstructured particles compared to the unstructured ones and tissue culture polystyrene. The expression of MGP was down regulated on the ceramics compared to tissue culture polystyrene. In contrast to the expression pattern on the ceramics at 48 h, the expression of MGP on microstructured BCP and HA was lower compared to the unstructured ceramics. The expression pattern of BMP2 was maintained after prolonged culture, with a higher expression on the microstructured BCP and TCP compared to the unstructured surfaces.

In conclusion, using a transcriptomics approach we identified five genes (HAS2, KIAA1199, ATF3, TNC and MGP) that have a consistent response to microstructural properties of calcium phosphate ceramics along a range of different material platforms and in different cell types, and confirmed this in an independent experiment.

Biological response to engineered microstructure in osteoinductive tricalcium phosphate ceramic discs

To further investigate and validate the effect of material properties on the expression of the GOI in the context of osteoinductivity, we produced a new set of materials, independent of the ceramic particles, based on a single chemistry with varying microstructural properties. Importantly, the TCP which was used in particle formulation in the previous experiments, is more osteoinductive than HA and BCP [12]. Knowing the effect of sintering temperature on the structural features (grain and pore size) of ceramics, we produced TCP based ceramic discs with three different temperatures (1150°C, 1050°C and 1000°C for TCPa, TCPb and TCPc, respectively). Material characterization confirmed that all three materials had the same chemistry (Figure S5) and that we successfully obtained a range of microstructural properties (Figure S6 and SEM images representing the surface are shown in Figure 5A). More specifically, the produced ceramics showed a range in grain size, pore size distribution, microporosity and specific surface area resulting in a variation in calcium and phosphate ion release (Figure S7). The grain sizes of these discs were smaller than the grain sizes of the microstructured HA and BCP. The microporosity and specific surface area of TCPc was higher than of the microstructured particles. In terms of pore size distribution, the TCP discs were similar to HAI and TCPp with a
mean pore size around 0.8 µm. A range in porosity was obtained and the measured pore volume of TCPa was similar to HAi, TCPb was similar to TCPp, and TCPc exhibited a higher incremental pore volume compared to all materials.

The expression of 12 genes, as identified in the aforementioned comparison based on microstructure, was analysed on the engineered TCP discs after 48 h (Figure 4). The expression levels of ATF3, MKNK2 and MGP were similar to the levels found for the unstructured materials (HAni and BCPni). However, consistent with the gradually increasing microstructural properties (grain size and microporosity) of the three TCP discs, only the expression of MGP exhibited a gradual (non-significant) trend. The expression on the discs was generally increased or decreased, in agreement with the direction identified in the microarray data, compared to the unstructured HAni and BCPni (HAS2, TNFRSF21, KIAA1199 and CEBPD). Moreover, differential expression compared to unstructured polystyrene was observed in the expression patterns of HAS2, KIAA1199, CEBPD, TNFRSF21 and MGP. The expression of TNC was higher on the discs compared to the two unstructured ceramic particles. Besides, the expression of BMP2, assessed in parallel to the identified “microstructural” GOI, did reveal a similar gradual increase to MGP. TCPc and TCPb, the discs with the smallest grain sizes and highest microporosity, induced a significantly higher expression than the unstructured HAni and BCPni particles.

Within the context of molecular responses to microstructural properties, HAS2, KIAA1199, CEBPD, TNFRSF21, and TNC were expressed at higher levels in cells on the TCP discs compared to the unstructured particles and polystyrene. Moreover, MGP and

Figure 5. Replication of ceramic surfaces in polystyrene. The left column of images represent the surfaces of three TCP discs sintered at varying temperatures (1150, 1050 and 1000 °C; a,b and c respectively) and a flat cyclic olyfinic polymer surface used as intermediate in the replication process. The right column of images depict the surfaces in polystyrene (PS) replicated using micropatterning tools (A). The grain sizes were measure on both the original ceramic surfaces and the imprinted polystyrene surfaces (B).
BMP2 expression profiles revealed a correlation to the microstructural properties of the ceramic discs.

**De-convolution of material properties**

The genes identified in this work are hypothesized to correlate with the microstructural properties of varying ceramics. These ceramic materials are complex systems with highly intertwined properties and concomitant biological effects. Consequently, differences in microstructure do not only refer to the surface properties but also influence the release of ions from the materials and specific protein adsorption. Because of the complexity of ceramics and the intertwined material properties, the effect induced by the range of microstructural properties (e.g. grain size, microporosity) of the TCP discs might have been masked by the higher dissolution properties. Therefore, to further unravel the origin of the observed gene expression differences, we attempted to simplify the ceramic system by de-convoluting the material properties to study the effect of surface structure and ion release separately.

To investigate the effect of the superficial microstructure of the ceramics, we optimized the replication of the surface morphology of TCP discs and a flat reference in polystyrene films using micropatterning tools (Figure 5A). Quantification of the grains on the imprinted surfaces revealed similar dimensions as the original ceramics (Figure 5B) which confirms a good replication using this processing technique and parameters. After confirming that these polystyrene imprints allow cell adhesion and growth (data not shown), gene expression of the GOI was assessed after 7 days of MG-63 culture (Figure 6). The small size of the imprint surfaces (0.8 mm in diameter) prevented gene expression assessment at earlier time points due to relatively low cell numbers. The expression of ATF3 exhibited a correlative trend with the microstructural properties of the imprinted PS. The expression increased from the flat PS to the one with the

**Figure 6. Gene expression on polystyrene mimicking ceramic surfaces.** Expression of ATF3 (A), HAS2 (B), TNC (C) and BMP2 (D) on flat PS, PSa, b and c (replicated from TCP a, b and c, respectively).
replicated surface of TCPa (PSa) and TCPb (PSb) until a statistically three fold increased expression when cultured on the replicated surface of TCPc (PSc) in comparison to the flat surface. However, according to the initial comparison based on the microarray between microstructured and unstructured ceramics, ATF3 expression was expected to decrease with smaller microstructural features. In contrast, the expression of TNC and HAS2 increased with the microstructural features. TNC was most affected with a gradual increase in expression paralleling the decreasing grain sizes from the flat PS surface to the microstructured imprints. TNC was significantly 4.5 fold up regulated on the smallest grain sized imprint compared to the flat surface. Similarly, the expression of HAS2 increased 2.8 fold. In addition, BMP2 did not respond in a clear way to the surface of the imprints, as only the imprinted surface of TCPc, with the smallest microstructural features, induced a two-fold up regulation compared to the flat surface. Overall, HAS2 and TNC from the GOI (excluding ATF3) seemed to be affected by the topographical cues from the ceramic discs presented in polystyrene.

While topographical cues play a key role in cellular responses to ceramics and biomaterials in general, the presence and the release of calcium and phosphate has
shown to greatly affect cell behaviour [4, 5, 7, 8, 45, 47]. We therefore addressed whether the correlation of the GOI with the osteoinductivity of ceramics is attributed to the effect of these two ions. MG-63 cells were cultured, in the absence of materials, in medium containing elevated levels of calcium (7.8 mM) and phosphate (10 mM), in comparison to non-supplemented medium (1.8 mM calcium and 1 mM phosphate). From the 12 initial genes tested, four were identified to be influenced by elevated levels of calcium and phosphate, namely KIAA1199, TNC, MGP and TNFRSF21 (Figure 7). Interestingly, the expression of KIAA1199 was strongly influenced after both two and seven days of culture with additional calcium and phosphate. In effect, these two ions separately down regulated the expression by approximately seven fold, whereas the combination of the two induced a 30 fold decrease after two days. Similarly, after seven days, exposure to phosphate reduced the expression levels 20 times in contrast to three and six times when exposed to calcium or the combination, respectively (Figure 7). The effect on TNC was milder, with a two to three fold up regulation in the varying conditions at both time points, similar to the effect size observed on the different ceramics. The expression of MGP was only affected after the prolonged exposure of seven days to phosphate, as a tenfold decrease was measured in response to this ion compared to basic concentrations, both in the presence of high and basal calcium levels. Finally, TNFRSF21 generally decreased three-fold after seven days of culture compared to two days for all but the combined condition. In other words, after seven days, the presence of both calcium and phosphate induced a three-fold up regulation of TNFRSF21 compared to the basic condition.

Furthermore, two genes (HAS2 and MKNK2) showed a five to ten fold decreased expression after seven days compared to two days of culture, independent of calcium and phosphate ion concentrations (Figure 7). This effect was also observed in osteogenic markers (BMP2, BGLAP and COL1A1; Figure S8). Indeed, the expression of BGLAP and COL1A1 after seven days were similarly down regulated compared to two days of culture. Only a negative effect of phosphate on COL1A1 expression was observed. Furthermore, a consistent up regulation of BMP2 was observed when MG-63 cells were exposed to combined high calcium and phosphate concentrations. The remaining genes (EGLN1, PPP1R3B, TPM1, ELL2 and ATF3) did not show any effects of varying concentrations of calcium or phosphate (data not shown). In conclusion, KIAA1199, TNC, MGP and TNFRSF21 were responsive to calcium or phosphate ions.

5.4 Discussion

The molecular mechanisms underlying osteoinductivity in correlation to the responsible material properties have yet to be defined. Therefore, gene expression profiling, which is not extensively applied in the field of biomaterials, was used in this study to address the cellular responses underlying cell-ceramic surface interactions [20, 35, 36, 39]. We successfully identified 13 genes with expression correlated to the microstructural and osteoinductive properties of ceramics, which was consistent in diverse cell types. The observed gene expression effects were reproduced in multiple independent experiments using different techniques and could be validated in different systems.

Osteoinductive ceramics possess the intrinsic capacity to induce bone formation by a specific combination of properties such as chemistry, surface microstructure and
consequent dissolution kinetics. To further study the expression of the 13 identified genes, beyond the HA, BCP and TCP-based ceramic particles with unstructured and microstructured surfaces, we engineered ceramic discs based on a single chemistry, tricalcium phosphate, with varying microstructural properties of interest. TCP has been shown to have a higher osteoinductive capacity but also a more pronounced dissolution kinetics [46, 48]. Although the expression of the majority of the 13 genes was higher on the TCP discs compared to microstructured HA and BCP, the expression values generally did not reflect the variations in surface structure. The tailored discs possess a rather small range of grain sizes compared to the particles (0.5-1.5 vs. 0.9-6.2 μm). Also, the discs were all microporous with a microporosity ranging from 20 to 45% in comparison to the particles, ranging from 0 to 45%. Concomitantly, the calcium release in SPS over time remained above 2 mg/dL whereas the release from the HA and BCP particles remained below 1 mg/dL. Because of the complexity of ceramics and the intertwined material properties, the effect induced by the range of microstructural properties (e.g. grain size, microporosity) might have been masked by the higher dissolution properties. Thereafter, to study in detail the GOI, we sought to simplify the in vitro model by de-convoluting the ceramic properties into surface structure and ion dissolution, which are suggested to be two crucial contributors to osteoinduction [2, 3, 5]. Using microfabrication techniques, we were able to replicate the microstructural features of ceramics in polystyrene films, which allowed us to study this material feature separate from the calcium phosphate ceramics. Moreover, the local ionic concentrations of calcium and phosphate upon dissolution from the ceramics have yet to be determined, so the proposed setup with elevated levels of both ions in the culture medium was used as a model system. Both separate model systems for surface structure and ion dissolution confirmed correlations between the GOI and the material properties.

The expression of HAS2 positively correlated to microstructure without an effect of elevated ion concentrations. HAS2 is a membrane bound enzyme responsible for the synthesis of hyaluronan within the skeleton [49]. In contrast to HAS1 and HAS3, HAS2 synthesizes the high molecular weight hyaluronan. High molecular weight hyaluronan, a non-sulfated glycosaminoglycan, can be found ubiquitously in all tissues where it plays a structural anti-inflammatory role. Of importance here is that hyaluronan is associated with multiple stages of endochondral bone formation and can influence osteoclast differentiation and bone nodule formation. Knockout mice lacking this particular enzyme exhibited abnormalities in their skeleton [50]. Hyaluronan synthesized and secreted by HAS2 in developing limb bud facilitates mesenchymal cell migration. The expression of its receptor, CD44, and their interaction play a role in pre-cartilaginous condensation [49]. KIAA1199, besides correlating to the microstructural properties of ceramics, was found to be drastically down regulated in response to elevated concentrations of calcium and phosphate. This protein, located in the cytoplasm, nucleus and endoplasmatic reticulum, is expressed in a wide range of tissues and has been reported to maintain cytosolic calcium ion homeostasis and to bind and depolymerize hyaluronan [51, 52]. Interestingly, KIAA1199 catabolizes hyaluronan via the clathrin-coated pit pathway, independently of the hyaluronidases and CD44 [52]. Moreover, whereas high molecular weight hyaluronan stimulates bone formation, its fragments inhibit it and suppress the expression of RUNX2 [53, 54].

The expression of TNC paralleled the microstructural properties of the materials in
both the particles and discs. Moreover, this gene was up regulated when MG-63 were cultured on polystyrene imprints with varying surface roughness and in response to both calcium and phosphate. Tenascin is a large extracellular matrix glycoprotein located in the peristeum and the endosteum. It is generally associated with sites of new tissue development and remodeling, and has been shown to be up regulated during osteoblast differentiation and in response to BMP2 [55]. However, a microarray study on MC3T3 stimulated with increased levels of inorganic phosphate reported a down regulation of TNC after 72 h together with other osteogenic ECM genes [8]. Similar to HAS2, condensing mesenchyme expresses TNC when undergoing chondrogenic differentiation. Moreover, the fact that mechanical strain/stress is known to induce the expression of TNC might explain the response to varying surface structures [56, 57].

The expression of matrix GLA (MGP) protein was up regulated in response to the microstructure of materials after 48 h whereas it was down regulated after 7 days. Moreover, MGP was highly down regulated after 7 days of exposure to phosphate. In contrast, other work showed that MGP is twofold up regulated in response to elevated levels of phosphate in the presence of 1.8 mM calcium after 24 h in MC3T3 mouse osteoblasts [58]. The MGP gene encodes a protein in the organic matrix of bone and cartilage and is a member of the mineral-binding GLA family of proteins that also includes osteocalcin, coagulation factors VII and IX. In fact, MGP binds intra- and extracellular minerals and ions and plays a role as a regulator of calcification. Interestingly, it has been shown to be a modulator of BMP2 and here the expression patterns of both genes were similar in the different material systems [59, 60]. Furthermore, the correlation between the other GOI and material properties (CEBPD, ATF3, MKNK2 and TNFRSF21), as evidenced by multiple microarray datasets and validation experiments, could not be attributed to

Figure 8. Proposed cellular mechanism in response to osteoinductive ceramics with an important role for cell-secreted ECM. HAS2 is expressed in response to surface microstructure whereas KIAA1199 is repressed in response to calcium and phosphate and by their combined action, high molecular weight hyaluronan is produced. Together with TNC, which is induced by the effect of both the surface microstructure and the ions, hyaluronan creates a niche that is conducive for an osteogenic phenotype and bone formation in vivo. Moreover, MGP is likely involved in this mechanism by regulating the extracellular matrix and osteoblast maturation.
separate material properties as assessed in the models systems used here. Possibly, these genes are affected via secondary material properties.

Based on the obtained results in this study, we propose a hypothetical molecular mechanism modulating the response to osteoinductive calcium phosphate ceramics, as schematically depicted in Figure 8. We hypothesize that osteoblasts cultured on microstructural ceramics express HAS2 in response to surface microstructure, and repress KIAA1199 expression in response to calcium and phosphate, and that the combined effect is responsible for an elevated production of hyaluronan. Together with TNC, which is induced by the effect of both the surface microstructure and the ions, hyaluronan creates a niche that is conducive for an osteogenic phenotype and bone formation in vivo. Moreover, MGP (together with BMP2) is likely involved in this mechanism by regulating the extracellular matrix and osteoblast maturation.

5.5 Conclusion

In conclusion, using a transcriptomic approach we identified 13 genes correlating to the osteoinductivity of calcium phosphate ceramics. To further unravel the mechanism leading to the observed differential expression we attributed the induced genes to separate material properties. On the one hand, using microfabrication tools to replicate the surface of ceramics in polystyrene, we confirmed that the expression of TNC and HAS2 is affected by surface microstructure. On the other hand, the expression of KIAA1199, MGP and TNC was affected by the addition of calcium and phosphate. The results suggest an important role for cell-secreted ECM in response to osteoinductive ceramics with an altered expression of HAS2, KIAA1199, TNC and MGP. We propose a mechanism by which the surface structure and ceramic-mediated ion release induce the expression TNC and MGP as ECM components and the expression of HAS2 and the repression of KIAA1199 modulating the molecular weight of the produced hyaluronic acid secreted in the ECM.
Chapter 5

5.6 References


Exploring cellular responses to osteoinductive ceramics

[58] Sweat A, Sane DC, Hutson SM, Wallin R. Matrix GlA protein (MGP) and bone morphogenetic protein-2 in aortic

5.7 **Supplementary figures**

**Figure S1.** Surface characterization of the studied calcium phosphate ceramics. The average size of the grains (A), microporosity (B), specific surface area (C) and pore size distribution (D) of the different ceramics.

**Figure S2.** Chemical characterization of the studied calcium phosphate ceramics. FTIR spectra (A) and XRD patterns (B) of the two HA, two BCP and TCPp.

**Figure S3.** Functional characterization of the studied calcium phosphate ceramics. Protein adsorption in 10% FBS containing medium after 2 h and 24 h, corrected for surface area of the ceramics (A). Cumulative calcium (B) and phosphate (C) release profile of the ceramics in simulated physiological saline.
Figure S4. Relative mRNA levels of TNC (A), KIAA1199 (B), MGP (C) and BMP2 (not an identified GOI) (D) after 7 days of culture on the different ceramics. ANOVA with Bonferroni correction was used to test the statistical significance of the expression between the non- and osteoinductive ceramics (* p < 0.05, ** p < 0.01, *** p < 0.001). ANOVA with Dunnett correction was used to test the statistical significance of the differential expression between the conditions and MG-63 cultured on tissue culture polystyrene (# p < 0.05, ## p < 0.01, ### p < 0.001).

Figure S5. Chemical characterization of the studied TCP discs. FTIR spectra (A) and XRD patterns (B) indicating the identical chemistry of TCPa, TCPb and TCPc.
Figure S6. Surface characterization of the studied TCP discs. The average size of the grains (A), microporosity (B), specific surface area (C) and pore size distribution (C) of the different ceramics.

Figure S7. Functional characterization of the studied TCP discs. Cumulative calcium (A) and phosphate (B) release profile of the ceramics in simulated physiological saline.

Figure S8. The expression of osteogenic marker genes in response to the addition of calcium and phosphate (in mM). Statistical significance between the two time points was assessed by two-way ANOVA with Bonferroni correction (* p < 0.05, *** p < 0.001).
## 5.8 Supplementary Tables

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Table S1. Primer sequences used to determine mRNA levels using qRT-PCR targeting the genes of interest (given with their ENSEMBL ID). Of note, no appropriate primers could be designed for RBPJ.
Chapter 6

High content imaging as a novel tool for automated analysis of biomaterial-induced cellular responses
Chapter 6
High content imaging as a novel tool for automated analysis of biomaterial-induced cellular responses

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Abstract
Upon contact with a biomaterial, cells and surrounding tissues respond in a manner dictated by the physicochemical and mechanical properties of the material. Traditionally, cellular responses are monitored using invasive analytical methods that report the expression of genes or proteins. These analytical methods involve assessing commonly used markers for a predefined readout, masking the actual situation induced in the cells. Hence, a broader expression profile of the cellular response should be envisioned, which technically limits up scaling to higher throughput systems. However, it is increasingly recognized that morphometric readouts, obtained non-invasively, such as cell shape are related to gene expression patterns. Here, we analyze the relation between the gene expression profiles and morphological characteristics of mesenchymal stromal cells when exposed to 4 materials with distinct surface roughness. Three PLA surfaces were modified by exposure to oxygen plasma of different duration times and the cellular response was compared to smooth untreated PLA surfaces without the addition of differentiation agents. Although the commonly used gene markers for osteo-, chondro- and adipogenesis did not allow the discrimination between the 4 different materials, the morphological and genome wide expression profile revealed underlying cellular changes. Using 3 morphometric parameters, obtained by high content imaging, we were able to build a classifier and discriminate between oxygen plasma-induced modified sheets and non-modified PLA sheets where evaluating classical candidates missed this effect. This approach indicates the feasibility to use noninvasive morphometric data in high-throughput systems to screen biomaterial surfaces indicating the underlying genetic biomaterial-induced changes.
6.1 Introduction

Recent progress in materials processing and chemical synthesis technology enables us to fabricate and synthesize biomaterials with virtually endless properties and compositions [1-4]. Furthermore, advances in micro- and nanotechnology have provided us with enabling technologies to perform high-throughput screening of a multitude of material characteristics and properties using cell-based readouts [5-8]. Similar to DNA microarray technology, these miniaturized platforms typically resemble an arrayed format for which technologies are being developed to achieve proper cell seeding and culturing. Another challenge in the field of high-throughput screening of biomaterials, designated materiomics, is to find the most efficient strategy to extract biological information from the cellular response to arrays of generated libraries. Traditionally, the field of biomaterials research uses a plethora of analytical techniques to report the expression of multiple molecular markers. Typically, in the case of mesenchymal stromal cells, one looks at osteogenic, chondrogenic or adipogenic differentiation pertaining to a given biomaterial behavior [9, 10]. This narrow view neglects other signaling pathways that may be involved in the differential response of cells to biomaterials. However, ideally, a broad picture is obtained indicating the overall response of cells to a certain material. For instance, gene expression profiling using DNA microarrays is able to provide a global insight in the cellular response. Unfortunately, to perform a DNA microarray analysis, a considerable amount of RNA needs to be extracted from the cells. On miniaturized biomaterial platforms the number of cells is often too low to isolate the required quantity of RNA. In addition, to probe a multitude of materials one needs arrayed isolation of thousands of RNA samples, which is technically not trivial. Therefore, microscopic analysis is currently the preferred strategy to screen material libraries [11, 12]. Obviously, the number of molecular markers that can be screened using fluorescent microscopy is limited to the number of available fluorescent channels on the high content bio-imaging equipment (nowadays typically four). Consequently, current assays for high content imaging focus on a relatively small subset of biological markers, even though the broader molecular context for cellular behavior is known. Therefore, it would be desirable to be able to use single cell morphometric analysis of cells growing on biomaterials and extract other relevant data related to cell shape or cytoskeletal conformation for instance that can be associated with the cellular behavior [13]. An interesting development within this respect is the evident link between morphological cellular parameters and complex biological responses of cells [14]. For instance, using light microscopy, staining of the cytoskeleton and staining of nuclei, images of individual cells under different experimental conditions can be captured [15]. Then, a morphological description comprising dozens of parameters can be compiled for individual cells using image analysis software [13, 16]. Next, using machine learning tools, morphological parameters that are predictive for the distinct experimental conditions, can be identified. For instance, morphological characteristics have been used to screen novel genes involved in the Rac signal transduction pathway [17]. In another study, morphological characteristics obtained using light microscopy of fertilized oocytes have been correlated to successful development into the blastocyst stage of development [18]. In the field of regenerative medicine, it has been demonstrated that it is possible to correlate the differentiation behavior of mesenchymal stromal cells using morphometric cytoskeletal parameters, which opens up the possibility to predict the behavior of mesenchymal stromal cells on a variety of biomaterial surfaces [13, 19].
In this manuscript, we aim to provide proof of principle for the next step, the possibility to evaluate cellular behavior using morphological data, by combining microarray-based functional network analysis with that of morphometric cytoskeletal parameters. A combination of such analyses allows us to identify how cellular morphometric parameters reflect the expression of certain genes from involved genetic networks. As model systems, we analyzed different surface roughness of Poly (DL-lactic acid) (PLA) sheets in comparison to smooth PLA surfaces. The surface roughness was introduced by applying different periods of oxygen plasma treatment. Mesenchymal stromal cells were cultured on the different sheets and two assays were performed; microarray analysis on one hand and fluorescence imaging-based single cell morphometric analysis on the other hand. We were able to demonstrate that cells cultured on non-treated smooth sheets differed significantly from cells cultured on plasma-treated substrates using both morphometric and microarray analysis.

6.2 Materials and Methods

Oxygen plasma treatment

300 µm thick poly (DL-lactic acid) (Folienwerk Wolfen GmbH) were treated with oxygen plasma using a SPI Plasma-Perp II (Structure Probe, Inc., West Chester, USA). A radio-frequency of 13.56 MHz, power of 100W, pressure around 200 µm (0.267 bar) was employed. The sheets were subjected to this treatment for 10, 20 and 30 minutes and the electrode was powered with a current of 100 mA.

Surface topography of PLA sheets was observed by scanning electron microscopy (SEM) with a Philips XL 30 ESEM-FEG. Samples were gold sputtered (Carringdon) before SEM analysis. Using SEM data, pit diameters were measured for at least 10 spots per image and 3 images per condition were used. Further analysis of surface roughness was carried out using atomic force microscopy (AFM). All the samples were fixed with double-sided tape to magnetic stubs. All the images were obtained at ambient conditions using tapping mode, with a force of about 50 N/m (TESP tip, Veeco), a scan area of 2 µm × 2 µm and a resolution of 256 × 256 pixels. A plane fit was applied to the image and surface roughness was calculated in Nanoscope V7.30. 3-D images were generated using SPIP™ software (Image metrology A/S, Denmark). Then, pit depths were measured using the profilometric tool in Nanoscope V7.30. Ten random locations were chosen for each image and at least 3 images per condition were used. Statistical analysis was done by one-way ANOVA followed by Tukey’s post-hoc test. Significance was set for p < 0.05.

Cell culture

Prior to cell seeding, the sheets were diced to fit 24-well non-treated tissue culture plates (Nunc) for morphometric analyses and 6-well non-treated tissue culture plates (Nunc) for gene expression studies. Each experiment was performed in triplicate. The diced sheets were subsequently placed in respective culture wells, secured using an O-ring (Eriks), sterilized using 70 % ethanol for 15 minutes and washed three times with phosphate buffered saline solution.
Bone marrow aspirate (5-20 ml) was obtained from a healthy donor with written informed consent. Human mesenchymal stromal cells (hMSCs) were isolated and expanded as described previously [20]. Briefly, the aspirate is resuspended using a 20-gauge needle, plated at a density of $5 \times 10^5$ cells/cm$^2$ and cultured in proliferation medium containing α-MEM (Gibco, 22-571-038), 10 % fetal bovine serum (FBS) (Lonza), 2 mM L-glutamine (Gibco, 25030), 0.2 mM ascorbic acid (Sigma, A8960), 100 U/ml penicillin + 100 g/ml streptomycin (Gibco, 15140-122) and 1 ng/ml basic fibroblast growth factor (bFGF) (Instruchemie, PhP105). Upon reaching confluence, the cells were trypsinized with 0.25 % Trypsin/EDTA solution. Cells were suspended in basic medium (proliferation medium without bFGF), seeded on the PLA sheets at a density of 5000 cells/cm$^2$ and cultured for 5 days in basic medium solely.

**Gene expression analysis**

After 5 days, total RNA was isolated from the cells cultured on different PLA sheets using a Nucleospin RNA II isolation kit (Macherey-Nagel) according to the manufacturer's instructions. The extracted total RNA was quantified spectrophotometrically using a ND 1000 spectrophotometer (Nanodrop technologies). mRNA levels from total RNA were determined by qPCR. cDNA was synthesized from 400 ng total RNA using iScript (Bio-Rad). 1 μl of undiluted cDNA was used for subsequent qPCR analysis which was performed on a Light Cycler real time PCR machine (Roche) using SYBR green I master mix (BioRad, Hercules, CA, USA). Data was analyzed using Light Cycler software version 3.5.3, using the fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of marker genes was normalized to GAPDH levels and fold inductions were calculated using the comparative ∆CT method [21] Primer sequences are provided in Table 1. Data were analyzed using one-way ANOVA followed by Tukey’s multiple comparison test; $p < 0.05$ was considered significant.

**Fluorescent imaging**

After culturing, cells on the different sheets were fixed with 4 % wt/v paraformaldehyde for 10 min, permeabilized with 0.1 % Triton X-100 and stained with Alexa Fluor 488 Phalloidin (Invitrogen) for the actin cytoskeleton and DAPI (Sigma) for nuclei. Images of cells were acquired automatically using a custom designed macro for a BD Pathway bioimager (BD Pathway 435, BD Biosciences). Briefly, the macro consisted of an autofocus algorithm based on the contrast of nuclear images. A set of two images, one for actin cytoskeleton and one for nuclei was acquired per sample. The images were automatically processed and analyzed using CellProfiler software. The CellProfiler pipeline consisted of measuring in total 555 different morphometric parameters such as area, perimeter, form factor, intensity and texture related to both nuclei and cytoskeleton for each cell. For these analyses, 48, 140, 108 and 106 hMSCs were measured for the non-treated and the 10, 20 and 30 min treated materials respectively. For the various surface modification comparisons, these morphometric parameters were ranked using a forward feature selection approach, using a logistic linear classifier to separate between non-treated and the 3 surface modifications.
Whole genome expression analysis

cRNA was synthesized from 400 ng RNA using the Illumina TotalPrep RNA amplification Kit (Ambion/Life Technologies), according to the manufacturer's protocol. Briefly, single stranded cDNA was synthesized using a T7 oligo (dT) primer followed by a second strand synthesis to obtain double stranded cDNA. Biotin-labeled cRNA was generated by in vitro transcription using T7 RNA polymerase. The RNA 6000 Nano assay was used to assess cRNA integrity on a Bioanalyzer 2100 (Agilent Technologies). Microarrays were performed using Illumina HT-12 v3 expression Beadchips (Illumina, Inc). First, 750 ng of cRNA was hybridized on each array overnight, then the arrays were washed and blocked followed by fluorescent signal development through addition of streptavidin Cy-3 (GE healthcare). Arrays were scanned on an Illumina iScan reader and analyzed with GenomeStudio (Illumina, Inc). For each type of surface treatment, 3 microarrays were performed (biological replicates). The measured raw intensity values were background corrected in BeadStudio (Illumina). Further data processing and statistical testing were performed using R and Bioconductor statistical software (http://www.bioconductor.org/). The probe-level raw intensity values were quantile normalized and transformed using variance stabilization (VSN). A linear modelling approach with empirical Bayesian methods, as implemented in Limma package [22], was applied for differential expression analysis of the resulting probe-level expression values.

<table>
<thead>
<tr>
<th>Osteogenic markers</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteocalcin</td>
<td>5’-GGCAGCGGATGTGAAGAG-3’</td>
<td>5’-GATGTTGTCAGCAACTG-3’</td>
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<tr>
<td>Osteopontin</td>
<td>5’-CCAACTAAGTCACGAAA-3’</td>
<td>5’-GGTGATGTCCTCTGTA-3’</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>5’-ACAGCATCCCACCTCAT-3’</td>
<td>5’-TTGACTCGTAACGTATGC-3’</td>
</tr>
<tr>
<td>Runx2</td>
<td>5’-GGAGTGGACGAGCAAGAT-3’</td>
<td>5’-AGCTTGCTGCTGCTTCTG-3’</td>
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</table>

<table>
<thead>
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<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5’-AGGCAATGTTGCTCCATT-3’</td>
<td>5’-GGCTTCCTGATCTATC-3’</td>
</tr>
<tr>
<td>Sox 9</td>
<td>5’-TGCTGAGCTTGCTGACCT-3’</td>
<td>5’-ATGGGGTGTCTTTTGT-3’</td>
</tr>
<tr>
<td>Collagen II</td>
<td>5’-CGTCAGATGACCTCCTAC-3’</td>
<td>5’-AGGCAAGGCTTCTGAG-3’</td>
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</table>

<table>
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<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ppary</td>
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<td>5’-GGATTCAGGTGTGCGATAC-3’</td>
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<table>
<thead>
<tr>
<th>Housekeeping gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’-CGCTCTGCTCGCTCTCGTT-3’</td>
<td>5’-CCATGGGTCTGACGATGT-3’</td>
</tr>
</tbody>
</table>

Table 1. Primer sequences used to determine mRNA levels using qRT-PCR
Chapter 6

The top 150 genes differentially expressed, having a p-value below 0.05, were considered for categorical analysis. Furthermore, a list of genes with p-values below 0.05, resulting in 2613 genes, was uploaded to the Ingenuity Pathway Analysis (IPA) knowledgebase (Ingenuity Systems) and tested for over-representation of canonical signalling pathways. Statistical analysis of over-representation of a pathway was performed using a right-tailed Fisher’s exact test. Pathways were ranked using these p-values and the top 25 was selected for functional genetic analysis of the treated surfaces [23].

6.3 Results and Discussion

Polymer surface modification by oxygen plasma treatment

hMSCs are able to respond to biomaterial surface topographies by changing both their morphology and gene expression profile. To investigate the correlation between both phenomena, we studied PLA sheets with different surface roughness modified using oxygen plasma treatment. Following oxygen plasma treatment, the PLA sheets were characterized using scanning electron microscopy (SEM) and atomic force microscopy (AFM). SEM helped us understanding the morphology of the modified surface and contact mode AFM allowed us to measure the pit depth. As can be seen from Figure 1 a-d different treatment times resulted in the modification of the surface of PLA sheets with variable degree of surface roughness. AFM data (Figure 1 e-j) further shows that upon higher exposure times of PLA to oxygen plasma, both the depth and mean feature diameter significantly increased. In conclusion, distinct material surface topologies and roughness was successfully produced by means of oxygen plasma treatment.

**Figure 1. Characterization of oxygen plasma treated PLA sheets.** A: representative SEM images of PLA sheet following 0, 10, 20 and 30 minutes of oxygen plasma treatment respectively (scale bar 5 µm). Note the dose dependent increase in surface roughness upon oxygen exposure. B: AFM images of PLA sheets following 0, 10, 20 and 30 minutes of oxygen plasma treatment, respectively. C: Average pit diameter on oxygen treated sheets, as determined using SEM images and average pit depth analysed on oxygen treated sheets as determined using AFM images. ** represents p < 0.05 and *** represents p < 0.01.
No correlation between candidate genes and surface modification

It is commonly known that materials are able to influence cellular behavior by virtue of different material properties, such as material elasticity \[24, 25\] or chemical composition \[26, 27\]. Consequently, the interest has grown into the development of materials with instructive properties circumventing the need for chemical additives to obtain a specific cellular phenotype. In this perspective, the ability of nano- or micro-scale topography to influence the osteogenic behavior of MSCs and osteoprogenitor cells has been reported \[8, 28, 29\]. Likewise, we observed changes in morphology of the hMSCs seeded onto the different plasma treated surfaces. To investigate underlying changes and whether the surface topography in our set of sheets was able to induce differential gene expression, we assessed the expression of specific markers for osteogenic, adipogenic and chondrogenic differentiation. We isolated RNA from hMSCs grown for 5 days on the PLA sheets produced with different surface roughness and performed qPCR analysis for genes involved in osteogenic differentiation such as alkaline phosphatase, runx2, and osteocalcin. None of these genes showed significant differences \((p > 0.05)\) in expression when cells cultured on the different PLA sheets were compared (Figure 2a). Similarly, no differences in expression of markers for chondrogenesis \((i.e. \text{Sox9}, \text{collagen II or aggrecan})\) or adipogenesis \((\text{PPARgamma})\) could be measured (Figure 2b and c). Apparently, the topography induced by oxygen plasma did not result in significant differential expression of these lineage-specific marker genes. It should be noted, however, that the experiment was not performed under differentiation circumstances. In effect no chemical additives were included in the medium so that solely the material properties were investigated by the cellular behavior which was analyzed in this paragraph through the expression of marker genes after 5 days of culture.

Figure 2. Marker gene expression in hMSCs cultured for 5 days on oxygen treated PLA sheets. A. Relative expression of osteogenic marker genes alkaline phosphatase, osteocalcin and runx2 in hMSCs on oxygen plasma treated PLA sheets compared to non-treated sheets. B. Relative expression levels of chondrogenic marker genes aggrecan, sox9 and collagen type 2. C. Relative expression levels of adipogenic marker gene PPAR gamma. All data were normalized to housekeeping GAPDH gene as described in the materials and methods section and with the expression in untreated sheets set to one. Statistical analysis was performed using ANOVA with Tukey's post testing but no significant differences \((p > 0.05)\) were observed.
Cellular morphometric analysis

An alternative strategy to investigate the response of a cell to a surface is by observing their morphology. To determine whether the different surface characteristics resulted in different cell morphologies; hMSCs were seeded on the different PLA surfaces and cultured for 5 days. Next, we stained the cells with phalloidin to visualize the actin cytoskeleton. As represented in Figure 3 it appeared to us that cells cultured on the non-treated PLA sheets presented a more fibroblastic, elongated appearance compared to cells cultured on sheets treated with oxygen plasma, which appeared to be spreading equally in all directions. Furthermore, the actin cytoskeleton appeared to be organized in more prominent stress fibers on oxygen plasma treated sheets, as can be observed by the structural and intensity differences in the fluorescently-labeled actin filaments between the different experimental conditions (Figure 3). Multiple cells were imaged per sheet and CellProfiler software allowed us to describe and quantify their morphological appearance (shown in Table 2, the definitions of the parameters are given in table 7). Indeed, we found statistically significant differences in cell morphology with cells on the oxygen treated surfaces having a lower perimeter and higher nucleus form factor, indicating that cells were indeed less elongated on oxygen treated surfaces. Using forward feature selection, we were able to readily distinguish cells growing on plasma treated and non-treated PLA sheets based on the top three selected morphological parameters being nuclear texture, intensity of actin cytoskeletal staining and the perimeter of the cells (see Figure 4). By performing 10 fold cross-validation, we determined that a logistic classifier based on the top 3 selected features (determined for every cross validation fold using forward feature selection), was able to correctly separate the cells growing on treated and non-treated materials with an accuracy of 0.92 (For 92 % of the cells, the classifier was able to correctly distinguish between cells from treated and non-treated surfaces). The AUC score (Area Under the Receiver Operating Characteristic (ROC) curve) [30] of the determined classifier is 0.89, reflecting the chance that it will correctly distinguish two cells randomly chosen from either type of surface. Apparently, these three cellular parameters are affected by the material surface roughness induced by the oxygen plasma treatment. To conclude, we are able to morphologically discriminate
High content imaging to screen biomaterials

between hMSCs grown on oxygen-treated versus non-treated PLA sheets. The difference in morphological appearance hints at differences in cytoskeletal organization.

The use of morphological features to interpret phenotypical changes is highly interesting for high-throughput screening applications as an alternative to invasive assays and extensive immunocytochemistry based screenings. However, this approach is not as specific as the use of molecular probes since the overall cellular morphology is observed instead of the molecular level. Nevertheless, the use of global morphological features is specific enough when screening for the cellular response and behavior for instance when screening for the dedifferentiation of chondrocytes, the change in morphology is as effective as collagen II staining [19].

<table>
<thead>
<tr>
<th>Parameter(^a)</th>
<th>Non-treated average</th>
<th>Treated average</th>
<th>P-value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell-based</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>28574</td>
<td>26284</td>
<td>0.51</td>
</tr>
<tr>
<td>Area/Nucleus Area</td>
<td>11</td>
<td>8.6</td>
<td>0.07</td>
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<tr>
<td>Eccentricity</td>
<td>0.85</td>
<td>0.84</td>
<td>0.91</td>
</tr>
<tr>
<td>Extent</td>
<td>0.41</td>
<td>0.42</td>
<td>0.71</td>
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<tr>
<td>Form Factor</td>
<td>0.12</td>
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<tr>
<td>Intensity (lower quartile)</td>
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<td>0.3</td>
<td>(1.98 \times 10^{-11})</td>
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<tr>
<td>Major Axis Length</td>
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<td>300</td>
<td>0.03</td>
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<tr>
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<tr>
<td>Orientation</td>
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<td>0.11</td>
</tr>
<tr>
<td>Perimeter</td>
<td>2342</td>
<td>1472</td>
<td>(8.83 \times 10^{-10})</td>
</tr>
<tr>
<td>Solidity</td>
<td>0.66</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Nucleus-based</strong></td>
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<tr>
<td>Area</td>
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<td>Extent</td>
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<td>Form Factor</td>
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<td>(2.56 \times 10^{-5})</td>
</tr>
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<tr>
<td>MinorAxisLength</td>
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<tr>
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<tr>
<td>Solidity</td>
<td>0.96</td>
<td>0.93</td>
<td>0.003</td>
</tr>
<tr>
<td>Texture (Inverse Difference Moment)</td>
<td>0.79</td>
<td>0.75</td>
<td>(2.89 \times 10^{-7})</td>
</tr>
</tbody>
</table>

\(^a\) Morphometric parameters were selected from a list of 555 parameters on their interpretability and uniqueness.\(^b\) P-values were determined using student’s t-test comparing the parameters retrieved from cells cultured on non-treated sheets with the 3 different treated sheets. After Bonferroni multiple testing correction for a 0.05 threshold, \(P\)-values < 0.002 can be considered significant. Significant \(p\)-values are italicized.

Table 2: Cell morphological analysis of hMSCs cultured on different PLA sheets.
Whole genome gene expression profiling

The change we observed in cell morphology as depicted above shows that the cells adapt to the surface they grow on, which is also likely to be reflected by changes in their gene expression profile. Previous publications have shown the interconnectivity between changes in morphology and cellular fate and behavior [13, 15, 19]. However, since candidate genes for osteo-, chondro- and adipogenesis did not expose this, we performed genome wide expression profiling using DNA microarray technology. To this end, a global overview of differential expressed genes between hMSCs grown on PLA sheets treated for 30 min versus non-treated PLA sheets was obtained. We analyzed the top 150 genes differentially expressed with a $p$-value below 0.05, on surfaces treated for 30 min with oxygen plasma. Interestingly, 24 of these top 150 genes were involved in cell adhesion, cell motility or actin cytoskeletal regulation, including for instance ACTC1, ITGA6 and several Keratins, indicating that the cells adapt their shape according to the surface they reside on (Table 3). Moreover, 12 differentially expressed genes of the top list were cartilage related or involved in chondrocyte extracellular matrix production (Table 4) such as COMP, COL10A1, HAS1 and DKK3 (Table 4). In Table 5, an overview is given of miscellaneous genes differentially regulated on the surface treated for 30 min. From this table, we observe a down regulation of genes involved in bone remodeling in cells grown on PLA sheets treated for 30 min.

Next, we analyzed the function of genes differentially regulated with a $p$-value lower than 0.05, resulting in 2613 genes, using Ingenuity Pathway analysis (Table 6). Interestingly, from the top 25 pathways in which these genes are involved, 4 relate to actin (top ranked) and integrin signaling (ranked fifth). Regulation of actin based motility by Rho and Integrin signaling were also pathways in the list of genes. These data confirm the

![Figure 4. Phenotypical analysis of hMSCs growing on PLA sheets. Graphical representation of morphometric discrimination between hMSCs cultured on the 3 treated PLA sheets versus non-treated PLA sheets. The separation is based on the top three morphometric parameters obtained using forward feature selection. The three morphometric parameters include "nucleus texture inverse difference moment", cell intensity (lower quantile) and "cell perimeter". The separation accuracy was 0.92, and the AUC score 0.89. Both scores were determined using 10-fold cross-validation.](image-url)
morphological response of MSCs to the different surface roughness induced by plasma treatment.

6.4 Conclusion

We have been able to evoke changes in the morphology of hMSCs by changing the surface properties of the PLA sheets on which the cells grew. Using fluorescent labeling, high-content imaging and forward feature selection, we were able to describe the morphology of the cells, based on three parameters, i.e. nuclear texture, intensity of actin cytoskeletal staining and the perimeter of the cells. The morphological data indicate a change in the cytoskeletal organization and may hint at the differential activity of signaling pathways.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Regulation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PODXL</td>
<td>2.38</td>
<td>0.0146</td>
</tr>
<tr>
<td>CD74</td>
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<td>0.0134</td>
</tr>
<tr>
<td>LOC400578</td>
<td>2.01</td>
<td>0.0069</td>
</tr>
<tr>
<td>MGC102966</td>
<td>1.98</td>
<td>0.0029</td>
</tr>
<tr>
<td>KRT14</td>
<td>1.88</td>
<td>0.0066</td>
</tr>
<tr>
<td>CSPG4</td>
<td>1.81</td>
<td>0.0008</td>
</tr>
<tr>
<td>FAP</td>
<td>-1.73</td>
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</tr>
<tr>
<td>MFGE8</td>
<td>1.64</td>
<td>0.0002</td>
</tr>
<tr>
<td>JAG1</td>
<td>1.62</td>
<td>0.0006</td>
</tr>
<tr>
<td>FRAS1</td>
<td>1.61</td>
<td>0.0025</td>
</tr>
<tr>
<td>KRT16</td>
<td>1.63</td>
<td>0.0043</td>
</tr>
<tr>
<td>PALM</td>
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<td>0.0179</td>
</tr>
<tr>
<td>LAMB1</td>
<td>-1.52</td>
<td>0.0252</td>
</tr>
<tr>
<td>ITGA6</td>
<td>1.51</td>
<td>0.0216</td>
</tr>
<tr>
<td>RRAS</td>
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<td>0.0059</td>
</tr>
<tr>
<td>SNTB1</td>
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<td>0.0291</td>
</tr>
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<td>KRT7</td>
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<td>0.0125</td>
</tr>
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<td>HES4</td>
<td>1.47</td>
<td>0.0113</td>
</tr>
<tr>
<td>ACTC1</td>
<td>1.47</td>
<td>0.0214</td>
</tr>
<tr>
<td>FLNB</td>
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<td>0.0003</td>
</tr>
<tr>
<td>CDH15</td>
<td>1.46</td>
<td>0.0007</td>
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<tr>
<td>RHOC</td>
<td>1.46</td>
<td>0.0133</td>
</tr>
<tr>
<td>EMILIN1</td>
<td>-1.43</td>
<td>0.0080</td>
</tr>
<tr>
<td>AFAP1</td>
<td>1.43</td>
<td>0.0023</td>
</tr>
</tbody>
</table>

\(^a\) Genes of interest retrieved from the top 150 gene list differentially regulated between oxygen-treated (30 min) and non-treated surfaces.  
\(^b\) Fold difference in expression between the two studied conditions. Positive fold regulating indicates an up regulation in oxygen-treated surfaces while a negative fold difference represents an up regulation in non-treated surfaces.  
\(^c\) p-values were determined using student's t-test, corresponding to the statistical significance of fold regulation between the two conditions.

Table 3. Cell adhesion and cytoskeleton organization related genes differentially regulated in plasma treated versus non-treated surfaces.
Nevertheless, qPCR analysis on osteogenic, chondrogenic and adipogenic markers did not reveal significant differences in gene expression. On the other hand, whole genome gene expression profiling identified differential expression of genes involved in cell adhesion and cytoskeletal organization and additionally identified differential regulation of genes related to cartilage and chondrocyte matrix production. Guided by the research shown in this manuscript, high content morphological imaging can be a powerful tool to screen libraries of biomaterials without the use of specific predefined markers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Regulation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMP</td>
<td>2.41</td>
<td>0.0364</td>
</tr>
<tr>
<td>CLEC3B</td>
<td>2.07</td>
<td>0.0134</td>
</tr>
<tr>
<td>COL10A1</td>
<td>-1.80</td>
<td>0.0007</td>
</tr>
<tr>
<td>ADM</td>
<td>-1.71</td>
<td>0.0159</td>
</tr>
<tr>
<td>CTHRC1</td>
<td>-1.72</td>
<td>0.0005</td>
</tr>
<tr>
<td>HAS1</td>
<td>1.67</td>
<td>0.0027</td>
</tr>
<tr>
<td>SI00A4</td>
<td>1.65</td>
<td>0.0429</td>
</tr>
<tr>
<td>STEAP1</td>
<td>-1.60</td>
<td>0.0267</td>
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<td>PTGES</td>
<td>1.53</td>
<td>0.0019</td>
</tr>
<tr>
<td>HAPLN3</td>
<td>1.50</td>
<td>0.0103</td>
</tr>
<tr>
<td>DKK3</td>
<td>1.45</td>
<td>0.0448</td>
</tr>
<tr>
<td>P4HTM</td>
<td>1.40</td>
<td>0.0037</td>
</tr>
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</table>

* Genes of interest retrieved from the top 150 gene list differentially regulated between oxygen-treated (30 min) and non-treated surfaces.

* Fold difference in expression between the two studied conditions. Positive fold regulating indicates an up regulation in oxygen-treated surfaces while a negative fold difference represents an up regulation in non-treated surfaces.

* p-values were determined using student’s t-test, corresponding to the statistical significance of fold regulation between the two conditions.

Table 4. Cartilage and cartilage matrix related genes differentially regulated in plasma treated versus non-treated surfaces.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name, Gene description</th>
<th>Fold Regulation</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLEC12</td>
<td>Collectin sub-family member 12. Scavenger receptor, binding antigens for host defense possesses collagen-like sequences.</td>
<td>-2.33</td>
<td>0.0290</td>
</tr>
<tr>
<td>CKB</td>
<td>Creatine kinase brain. Cytoplasmic enzyme involved in energy homeostasis, reversibly catalyzes the transfer of phosphate from/to ATP.</td>
<td>2.21</td>
<td>0.0011</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid beta (A4) precursor protein. Brain related cell surface receptor and transmembrane precursor protein.</td>
<td>-2.17</td>
<td>0.0274</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E. Scavenger of lipoproteins in blood and brain.</td>
<td>-2.11</td>
<td>0.0177</td>
</tr>
<tr>
<td>MYL9</td>
<td>Myosin light chain 9, regulatory. Regulates muscle contraction.</td>
<td>-2.05</td>
<td>0.0176</td>
</tr>
<tr>
<td>PENK</td>
<td>Proenkephalin. Neuropeptide hormone activity.</td>
<td>2.02</td>
<td>0.0038</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase muscle. Glycolysis, catalyzes phosphoryl group transfer to ADP.</td>
<td>-1.98</td>
<td>0.0202</td>
</tr>
<tr>
<td>C10orf116</td>
<td>Chromosome 10 open reading frame 116 (Adipose specific 2). Function unknown exclusively expressed in adipose tissue.</td>
<td>1.93</td>
<td>0.0006</td>
</tr>
<tr>
<td>WISP2</td>
<td>WNTI inducible signaling pathway protein 2. Possible role in modulating bone turnover.</td>
<td>-1.74</td>
<td>0.0079</td>
</tr>
<tr>
<td>IAK1</td>
<td>Janus kinase 1. Protein-tyrosine kinase involved in signal transduction pathways.</td>
<td>-1.73</td>
<td>0.0244</td>
</tr>
<tr>
<td>PPP1R3C</td>
<td>Protein phosphatase 1, regulatory subunit 3C. Catalyzes reversible protein phosphorylation.</td>
<td>1.60</td>
<td>0.0087</td>
</tr>
<tr>
<td>SMAD6</td>
<td>SMAD family member 6. Negatively regulates BMP and TGF-beta/activin signaling.</td>
<td>1.58</td>
<td>0.0015</td>
</tr>
<tr>
<td>MYH11</td>
<td>Myosin heavy chain 11, smooth muscle. Functions as a contractile protein.</td>
<td>1.55</td>
<td>0.0011</td>
</tr>
<tr>
<td>CTSK</td>
<td>Homo sapiens cathespin K. Involved in bone remodeling and resorption.</td>
<td>-1.54</td>
<td>0.0079</td>
</tr>
<tr>
<td>MKD</td>
<td>Midkine (neurite growth-promoting factor 2). Promotes angiogenesis.</td>
<td>-1.52</td>
<td>0.0028</td>
</tr>
<tr>
<td>CD248</td>
<td>CD248 molecule (Endosialin). Related to tumorangiogenesis.</td>
<td>1.49</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

a Genes of interest retrieved from the top 150 gene list differentially regulated between oxygen-treated (30 min) and non-treated surfaces.

b Fold difference in expression between the two studied conditions. Positive fold regulating indicates an up regulation in oxygen treated surfaces while a negative fold difference represents an up regulation in non-treated surfaces.

c P-values were determined using student’s t-test, corresponding to the statistical significance of fold regulation between the two conditions.

Table 5. Miscellaneous genes of interest differentially regulated in plasma treated versus non-treated surfaces.
### Table 6. Top 25 list of Ingenuity Pathway analysis of genes differentially expressed between oxygen plasma treated and non-treated surfaces

<table>
<thead>
<tr>
<th>Ingenuity Canonical Signaling Pathways</th>
<th>P-Value(^b)</th>
<th>Ratio(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin Cytoskeleton Signaling</td>
<td>0.0006</td>
<td>0.173</td>
</tr>
<tr>
<td>Role of JAK1 and JAK3 in γ Cytokine Signaling</td>
<td>0.0007</td>
<td>0.246</td>
</tr>
<tr>
<td>IL-9 Signaling</td>
<td>0.0009</td>
<td>0.289</td>
</tr>
<tr>
<td>Agrin Interactions at Neuromuscular Junction</td>
<td>0.0009</td>
<td>0.254</td>
</tr>
<tr>
<td>ILK Signaling</td>
<td>0.0010</td>
<td>0.187</td>
</tr>
<tr>
<td>p70S6K Signaling</td>
<td>0.0011</td>
<td>0.205</td>
</tr>
<tr>
<td>Mitochondrial Dysfunction</td>
<td>0.0016</td>
<td>0.188</td>
</tr>
<tr>
<td>Germ Cell-Sertoli Cell Junction signaling</td>
<td>0.0019</td>
<td>0.189</td>
</tr>
<tr>
<td>Virus Entry via Endocytic Pathways</td>
<td>0.0041</td>
<td>0.207</td>
</tr>
<tr>
<td>Regulation of eIF4 and p70S6K Signaling</td>
<td>0.0052</td>
<td>0.174</td>
</tr>
<tr>
<td>Interferon Signaling</td>
<td>0.0071</td>
<td>0.265</td>
</tr>
<tr>
<td>AMPK Signaling</td>
<td>0.0087</td>
<td>0.173</td>
</tr>
<tr>
<td>IGF-1 Signaling</td>
<td>0.0095</td>
<td>0.186</td>
</tr>
<tr>
<td>Myc Mediated Apoptosis Signaling</td>
<td>0.0107</td>
<td>0.217</td>
</tr>
<tr>
<td>Small Cell Lung Cancer Signaling</td>
<td>0.0112</td>
<td>0.179</td>
</tr>
<tr>
<td>EGF Signaling</td>
<td>0.0112</td>
<td>0.229</td>
</tr>
<tr>
<td>ERF2 Signaling</td>
<td>0.0151</td>
<td>0.176</td>
</tr>
<tr>
<td>JAK/Stat Signaling</td>
<td>0.0162</td>
<td>0.203</td>
</tr>
<tr>
<td>Molecular Mechanisms of Cancer</td>
<td>0.0182</td>
<td>0.139</td>
</tr>
<tr>
<td>Regulation of Actin-based Motility by Rho</td>
<td>0.0186</td>
<td>0.184</td>
</tr>
<tr>
<td>Integrin Signaling</td>
<td>0.0219</td>
<td>0.151</td>
</tr>
<tr>
<td>Docosahexaenoic Acid (DHA) Signaling</td>
<td>0.0219</td>
<td>0.225</td>
</tr>
<tr>
<td>Role of JAK2 in Hormone-like Cytokine Signaling</td>
<td>0.0219</td>
<td>0.229</td>
</tr>
<tr>
<td>IL-4 Signaling</td>
<td>0.0234</td>
<td>0.188</td>
</tr>
<tr>
<td>Semaphorin Signaling in Neurons</td>
<td>0.0240</td>
<td>0.212</td>
</tr>
</tbody>
</table>

\(^a\) Uncorrected p-value < 0.05, resulting in a list with 2613 genes

\(^b\) P-Value calculated based on the Right-tailed Fisher's Exact test, defining the statistical significance of over-representation of the pathway in the gene list compared to a reference gene list

\(^c\) Ratio is the number of genes from the gene list that participate in the canonical signalling pathway divided by the total number of genes involved in the pathway.
### Table 7. Overview of features determined by CellProfiler

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intensity based</strong></td>
<td></td>
</tr>
<tr>
<td>Intensity (lower quartile)</td>
<td>The intensity value of the pixel for which 25% of the pixels in the object have lower values.</td>
</tr>
<tr>
<td><strong>Shape based</strong></td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>The actual number of pixels in the region.</td>
</tr>
<tr>
<td>Area/Nucleus Area</td>
<td>The eccentricity of the ellipse that has the same second-moments as the region.</td>
</tr>
<tr>
<td>Eccentricity</td>
<td>The eccentricity is the ratio of the distance between the foci of the ellipse and its major axis length. The value is between 0 and 1. (0 and 1 are degenerate cases; an ellipse whose eccentricity is 0 is actually a circle, while an ellipse whose eccentricity is 1 is a line segment.) This property is supported only for 2D input label matrices.</td>
</tr>
<tr>
<td>Extent</td>
<td>The proportion of the pixels in the bounding box that are also in the region. Computed as the Area divided by the area of the bounding box.</td>
</tr>
<tr>
<td>Form Factor</td>
<td>Defined as 4<em>n</em>π*Area/Perimeter^2. Equals 1 for a perfectly circular object.</td>
</tr>
<tr>
<td>Major Axis Length</td>
<td>The length (in pixels) of the major axis of the ellipse that has the same normalized second central moments as the region.</td>
</tr>
<tr>
<td>Minor Axis Length</td>
<td>The length (in pixels) of the minor axis of the ellipse that has the same normalized second central moments as the region.</td>
</tr>
<tr>
<td>Orientation</td>
<td>The angle (in degrees ranging from -90 to 90 degrees) between the x-axis and the major axis of the ellipse that has the same</td>
</tr>
<tr>
<td>Perimeter</td>
<td>The total number of pixels around the boundary of each region in the image.</td>
</tr>
<tr>
<td>Solidity</td>
<td>The proportion of the pixels in the convex hull that are also in the region. Also known as convexity. Computed as Area/Convex Area.</td>
</tr>
<tr>
<td><strong>Texture based</strong></td>
<td></td>
</tr>
<tr>
<td>Texture (Inverse Difference Moment)</td>
<td>Texture features are derived from a co-occurrence matrix, which contains information about how image intensities in pixels with a certain position in relation to each other occur together. For example, how often does a pixel with intensity 0.12 have a neighbor 2 pixel to the right with intensity 0.15? It can measure textures at different scales; the scales you choose determine how the co-occurrence matrix is constructed. For example, if you choose a scale of 2, each pixel in the image (excluding some border pixels) will be compared against the one that is two pixels to the right. The features are based on the Haralick features described by Haralick et al [20].</td>
</tr>
</tbody>
</table>

* Interpretation of the parameters analyzed and mentioned in this manuscript as provided by CellProfiler.
6.5 References


Chapter 7

Reflections and future outlook: Considering complexities
Chapter 7

Reflections and future outlook: Considering complexities

Nathalie Groen
7.1 Introduction

Regenerative medicine requires an understanding of the complexity of life science principles and biomaterials for the successful development of new therapeutic approaches. The biomaterial field has evolved over past decades witnessing important successes using the classical design and engineering approach. However, the scientific developments leading to clinical translation could be more efficient and rapid. For the field to undergo this transition, the scientific complexity at both sides of the interface – the material on the one hand and the organism on the other – needs to be considered. Both synthetic and biological complexity can be addressed using similar approaches; in this chapter our envisioned approach herein is discussed emphasizing how this thesis contributes to this approach in the context of bone tissue engineering and regeneration applications.

7.2 Classical biomaterial research

Classically, biomaterial research is largely confined to a low-throughput and iterative experimentation approach to develop a biomaterial suitable for a specific application. Using this ‘trial and error’ methodology, clinically applied biomaterials have been successfully developed such as coronary stents, orthopaedic implants or intraocular lenses. However, the exponentially grown body of scientific work on biomaterials is not proportional to the low number of clinical successes. Typically, a biomaterial is subject to iterative engineering, at different property scales (e.g. chemistry, structural or mechanical), driven by hypotheses emerging from biological concepts or practical intuition. For instance, scientists argue that in vitro cell phenotype is more functional when the natural in vivo extracellular matrix is structurally mimicked using electrospinning techniques [1] or that osteoblastic function is enhanced on rougher surfaces because it mimicks that of osteoclastic resorption during bone remodeling [2-4]. The inevitable consequence of trial and error approaches is that variations to one property alter other properties, yet at different scales. Exemplary is the iterative design of osteoinductive materials over the past 25 years, as properties enabling osteoinduction are (still) undefined. Doing so, the tridimensional macrostructure, interconnected pore structure and surface microtopography have all shown (separately or in combination) their valuable contribution to the osteoinductivity of calcium phosphate ceramics [5-8]. However, attempts at improving the mechanical properties of osteoinductive ceramics resulted in a decrease in the osteoinductive capacity.

Importantly, adequate in vitro models to test biomaterials are still largely lacking. In vitro assays characterizing the biological effect of designed biomaterials are similarly inspired by biology yet based on intuition rather than proven effectiveness. Biomaterials for bone regeneration applications are mostly characterized using in vitro assays without proven predictable efficacy such as osteogenic differentiation to address bone forming capacity of cells or materials [9-11]. That being said, advances in developmental and (stem) cell biology may provide a solid basis for hypothesis-based biomaterial research. Candidate approaches have indeed been successful. The in vivo bone forming capacity of human mesenchymal stem cells (MSCs) was enhanced by specifically targeting protein kinase A signalling, selected based on its reported implications in vascular calcification and bone mineral density [12]. Alternatively, when targets for aimed signalling pathways
Reflections and future outlook

are unknown, a high-throughput screening approach can be employed to for example identify small molecules mimicking hypoxia to modulate angiogenic responses in MSCs [13]. Besides, *in vitro* cell based assays often involve assessing a whole population using for instance qRT-PCR techniques or ELISAs, where valuable single cell, subpopulation or spatial information, obtained by *e.g.* staining or flow cytometry, is lost.

As aforementioned, the above described approach has led to successful clinical translation to some extent. Specifically, within the scope of this thesis, a few calcium phosphate based products are available on the market for orthopeadic or maxillofacial applications, however, for minimal weight baring applications. Also, the available products are mainly, but not exclusively, osteoconductive and progresses achieved with osteoinductive materials still have to reach the clinic [14, 15]. Clearly, the biomaterials field requires a different approach to enable further, more effective and rapid developments. Considering the complexity of both biological and material systems seems a fundamental necessity herein. Successful engineering of biomaterials for biomedical purposes might need a reverse engineering approach in order to decompose and understand the biological systems.

7.3 Complexity

Tissue engineering aims at implanting a temporary scaffold that permits and promotes regeneration of the damaged tissue through stimulation of the body to heal itself. Therefore, the intrinsic complexity of biological systems is a crucial concept to acknowledge; do not underestimate nature. Nature has built ‘diversity’, complex systems, using smart combinations of relatively few ‘universal’ components at different hierarchical scales. Using a small amount of building blocks, either chemical elements or four nucleotides, nature has created structural and functional diversity. Unraveling this complexity (*e.g.* ENCODE project; identifying all functional elements in the genome) at one single scale already seems a meticulous and infinite job.

Similarly, a (synthetic or natural) material should be considered as a complex system composed of a hierarchical combination of properties (from elemental, via structural to functional). An osteoinductive calcium phosphate ceramic is hierarchically composed of, naturally, calcium and phosphate, a crystal structure, a surface charge, grain and pore sizes, surface topography, microporosity and all together with its macroporosity determine its final clinical effectiveness in inducing and promoting bone formation. As such, improving the mechanical properties of osteoinductive ceramics by the addition of mechanically more stable polymers is successful in improving the mechanical properties but it is at the expense of the osteoinductivity. Not only are different properties interrelated to and influencing each other, yet at different scales, also different properties might converge to the same biological effect. For example, in chapter 5, we observed that tenascin-C, upregulated on ceramic materials, was responsive to both microstructural properties and to calcium and phosphate ions separately.

The challenge to design smart materials to regenerate biological tissues is intrinsically double-sided, yet highly intertwined. The scientific complexity at both sides of the interface – the material on the one hand and the organism on the other – needs to be considered. Such multi-scale complexities make any single-scale analysis and prediction a hypothesis at best and hence ask for a multi-scale approach.
7.4 Omics approach

The double-sided complexity hampering developments in tissue engineering can potentially be tackled using a holistic approach, that is an ‘omics’ approach. Omics, by definition, refers to “all constituents considered collectively”. This type of approach is not new: the pharmacological field integrated combinatorial synthesis, high-throughput screening and computational modeling to upscale and advance drug discovery many years ago. Other fields similarly benefitted from this combinatorial approach (e.g. semiconductors and coatings [16, 17]). This approach could be applied in any field and indeed has been proposed to bring the biomaterials field a step ahead [18-21].

Materiomics to tackle material complexity

Materiomics works from this philosophy of convergence, and is characterized by a materials science approach that considers all mechanisms of a material system across multiple scales. Using this approach the complexity from the material side has been addressed. Typically, the structure-performance correlation is the key to either bottom up or top-down approaches. On the one hand, these correlations help the simple design, mimicking from nature, of materials from the smallest element. On the other hand, the correlations help modeling the multi-scale properties from a material as a holistic approach. Either way, it contributes to understanding material properties.

These structure-function correlations are typically addressed using high-throughput screening approaches; either assessing the structural effect of a multitude of combined elements or assessing the structural effect of a library of materials with varying characterized properties [22-24]. These structure-performance readouts are indicative on the one hand about the functional (e.g. biological) performance, and serve as input for modeling other correlations and consequently new designs on the other hand. High-throughput approaches are the key in this process. Indeed, libraries of varying materials emerged; for example libraries of polymers with varying chemistries, physical properties or surface topographies [25-28].

Hitherto, employed assays to determine protein adsorption, rate and mechanisms of degradation, cytotoxicity, biocompatibility and cellular proliferation are examples. Although these assays may be tedious, most of them enable high-throughput screening and support modeling of property-performance questions. However, what do these readouts tell us about the in vivo biological functionality of materials?

Genomics to tackle biological complexity

Specialized in vitro tests to determine in vivo biomaterial performances are still largely lacking. This is a crucial step in testing anything; without an appropriate readout or biological hypothesis, the evaluation of materials and the effect of their properties are meaningless. The necessity of in vivo assessment to test the osteoinductivity of a material for bone regeneration applications for example results from poorly predictive in vitro models. So far we were not able to assemble, simplify or predict the biological complexity
of tissues in an in vitro model. We therefore need to better understand the biology of the tissue of interest. Concepts from developmental biology have been used, for instance in the field of cartilage repair. In the case the intended tissue has regenerative capacities, the mechanisms during healing may be studied and used. Alternatively, we may use indirect correlations, i.e. correlating in vitro behavior on materials with known in vivo performances. However, the convenient existence of a successful material with known in vivo application is herein a prerequisite.

In order to unite the biological complexity in an in vitro model, we propose the use of another ‘omics approach: transcriptomics. Historically, gene expression profiling has been useful to unveil biological signalling pathways, study mechanisms involved in development or to study stem cell niches. Furthermore, genomics is commonly used in the pharmacological field to investigate the effect of therapeutics and small molecules. The gene expression profile represents, via a global measurement, the cell's transcriptional state; giving a clue about its response to external factors and hinting towards its phenotype. External stimuli from surrounding cells and extracellular matrix substantially define cellular identity and behavior (e.g. differentiation) via the gene expression profile of each cell. Indeed, a hypothesis to test biomaterials may be defined evolving from biological systems studied using gene expression profiling.

In tackling the biological complexity, gene expression profiling is not only a useful tool to study and understand the biology of the intended tissue but also to monitor the biological effects of a biomaterial (both in vivo and in vitro). The actual in vitro model may use gene expression profiling to characterize the cellular response to a biomaterial. In fact, whole cellular profiles may reveal additional information otherwise hidden when a specific biological readout of interest (e.g. BMP2 up regulation or secretion) is assessed. For instance, in chapter 4, the dramatic gene expression changes observed between polymeric/ceramic composites treated with different concentrations of NaOH were not depicted in the expression patterns of osteogenic markers genes and as such may be missed.

**Genomics to improve osteoinductive materials**

As aforementioned, the field of biomaterials for bone regeneration would greatly benefit from predictive in vitro models to test osteoinductivity. In vivo biomaterial-induced bone formation is a discovered phenomenon of which the underlying biological mechanism is poorly understood. This lack hampers the improvement of osteoinductive materials for bone regeneration applications, that is, advancements are certainly being made but rather at low pace. As postulated in the previous paragraph, one might use a candidate approach targeting mechanisms involved in bone biology, inspired from development or bone healing. With reference to bone formation, the osteogenic differentiation behavior (e.g. ALP activity or expression of osteogenic markers) of osteoblastic precursor cells on biomaterials is typically evaluated to address the osteoinductive capacity. This approach is often applied in biomaterial research, however, not in the scope of developing an in vitro model but to evaluate and characterize the material of interest. Indeed, in vitro models have to be validated; is the used model predictive for the in vivo capacity?
Another possible approach is enabled by the existence of osteoinductive ceramics which have been studied for several years. Gene expression profiling of \textit{in vivo} tissue reaction over time might unveil the molecular mechanisms occurring upon implantation of an osteoinductive ceramic. Alternatively, an indirect approach may be employed (such as in chapter 5) that uses the correlation between the \textit{in vitro} gene expression profiles of a single cell type cultured on different ceramics and the \textit{in vivo} osteoinductivity. This reveals insights in the induced molecular mechanism which may be in turn to develop an \textit{in vitro} assay.

\subsection*{7.5 Converge-omics}

Both abovementioned omics approaches, materiomics and transcriptomics are already being used separately in several fields including the biomaterials field. However, to pursue the advancements in the biomaterials field \textit{converging multiple omics} fields may present the key to success by addressing the double-sided complexity presented by biomaterials for tissue regeneration. Therefore, the convergence of the two omics fields seems a natural choice with materiomics dealing with the complexity presented by material systems and with genomics addressing the biological complexity at gene expression level. As such, using a systematic approach, libraries containing a large variety of materials properties on the one hand and corresponding genome-wide gene expression profiles on the other hand can be built. Such libraries with high throughput data on both scales (biological and material) would allow powerful correlation analyses to define the biological functionality of material properties and as such helps understanding and improving materials for biological applications. Importantly and as mentioned before, an \textit{in vitro} model with a biological question is crucial when addressing these libraries. Without any biological readout correlations between material properties and gene expression profiles are endless and meaningless. Also, integration of multi-scale experimental analyses may present the future key to improve our understanding of how structure and properties are linked as most properties (biological and material) are strongly dependent on the scale of observation.

An example of convergence of multiple omics approaches is the Connectivity map \cite{29, 30}. It assembled the transcriptional profiles of cells exposed to a large set of bioactive molecules. The response to the compounds is reflected in the transcriptional profiles of the cultured cells. High throughput screening of compounds not only enables to identify hit compounds with aimed effects but also to define structural-functional relationships. Indeed connections between molecules sharing a molecular response allow to explore the chemical-functional relationships thereby modeling and designing molecules with improved biological properties.

With the emergence of \textit{in situ} RNA sequencing, genomics is brought to single cell scale acquiring a spatio-temporal dimension \cite{31}. This approach combines imaging techniques with RNA probes, allowing so far to assess 60 genes simultaneously. However, major developments are necessary to upscale the detection of transcripts. This method would allow for instance to address the transcriptional responses of all different cells in the vicinity of an implanted material (\textit{e.g.} ceramic) to understand the biological responses, which may be masked when using a population-wide tool such as gene expression profiling. Moreover, other omics fields could be used alongside or instead of
genomics to tackle the biological complexity. Proteomics, for instance, analyses the entire set of proteins translated or modified by a biological system. Otherwise, morphological and cytoskeletal descriptors may be used to screen materials (for example in chapter 6) [32, 33]. However, unless correlated to a biological functionality, cytoskeletal and morphological parameters do not directly reveal a biological functionality [34]. This imaging-based approach allows for live-monitoring where gene expression profiling demands an end-point measurement via cell lysis. Naturally, antibody-based screening targeting a protein of interest is a functional readout, however, belongs to the candidate rather than holistic approaches.

7.6 Conclusion

In our opinion, combining multiple omics approaches to address both the biological and the material complexity simultaneously would help advancements in the biomaterials field for tissue regeneration applications. Not only new insights in biology should be obtained in order to adequately address the effect of material properties but also full cellular response assessments are necessary. Moreover, the different omics fields require a collaboration between experts in their respective fields; without material sciences, biological knowledge or bioinformatics analyses, the proposed approach is not viable. Indeed, the multidisciplinary required for tissue engineering is even more true.
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Reflections and future outlook


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Biomaterials. 2011 Sep;32(26):6089-98.
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I promised myself a long time ago that I would write this important section of this book with a lot of care and attention and of course well in advance. Well, that failed… If these are the first words you read in this thesis; please know that I would like to thank you!

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Nathalie