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Interrogating the osteogenic potential of implant surfaces *in vitro*; A review of current assays

Avery SJ, Ayre WN, Sloan AJ, Waddington RJ

Steven James Avery, PhD, School of Dentistry, College of Biomedical and Life Sciences / Cardiff Institute for Tissue Engineering and Repair, Cardiff University, Heath Park, Cardiff, UK; phone +44 (0)1453 524591; email Steven.Avery@Renishaw.com.

Wayne Nishio Ayre, PhD, School of Dentistry, College of Biomedical and Life Sciences / Cardiff Institute for Tissue Engineering and Repair, Cardiff University, Heath Park, Cardiff, UK; phone +44 (0)29 225 10 660; email AyreWN@cardiff.ac.uk.

Alastair James Sloan, PhD School of Dentistry, College of Biomedical and Life Sciences / Cardiff Institute for Tissue Engineering and Repair, Cardiff University, Heath Park, Cardiff, UK; phone +44 (0)29 225 10650; email SloanAJ@cardiff.ac.uk.

Rachel Jane Waddington, PhD, School of Dentistry, College of Biomedical and Life Sciences / Cardiff Institute for Tissue Engineering and Repair, Cardiff University, Heath Park, Cardiff, UK; phone +44 (0)29 225 10647; email WaddingtonRJ@cardiff.ac.uk.

Running title: *In vitro* analysis of osteogenesis

*** Corresponding Author;** Professor Rachel Waddington, Oral and Biomedical Sciences, School of Dentistry, Heath Park, Cardiff University, Cardiff, CF14 4XY, UK.

Tel: +44(0)29255 10647; email: waddingtonrj@cardiff.ac.uk

Abstract

The success of implantable devices relies heavily on their interaction with the host cells facilitating the osteointegration process. However, with so many new surface modifications, with subtly varying design parameters, *in vitro* assays can, with proper interpretation, provide valuable information for understanding cellular behaviour. This review brings together pertinent *in vitro* experimental protocols available to researchers and discusses them in relation to the development of the osteoblast phenotype during bone repair. Consideration is also paid to the influence of endothelial and macrophage cells that can substantially change osteogenic cell activity and thus can provide added value for predicting the osseointegration potential *in vivo*. Due to the diverse and heterogeneous nature of cell types available for culture use, this review concludes there is no “gold standard” series of assays. Rather we present guidance in the experimental design of *in vitro* assays to better identify those surfaces with promising osteogenic potential.

Key words

implants, osteointegration, bone repair, osteogenesis, bone marrow stromal cells, angiogenesis, endothelial cells, macrophages

Impact statement

Titanium implants are already widely used in orthopaedics and dentistry, yet intensive research continues with the aim of modifying and functionalising implant surfaces to invoke a stronger bone response and to meet current clinical challenges around improving longevity, decreasing morbidity, widening access and clinical application. A very large number of surface modifications have been studied and the potential for new designs appears to be limitless as new technology grows. This review provides guidance for *in vitro* assays available to test these technologies, providing a cost-effective means for acquiring robust and physiologically relevant data, prior to *in vivo* examination.

Introduction

Titanium implantable devices have provided a successful treatment option in many areas of reconstructive orthopaedic and cranioplasty medicine and dentistry. However, despite these clinical successes, many challenges for their use remain. These include increasing the longevity of the implant and widening their use to age-related conditions where bone quality is reduced, such as osteoporosis and type 2 diabetes mellitus¹. For these reasons, research efforts look to develop novel implant surfaces that can rapidly and effectively integrate within host bone tissue. However, in researching these metals a plethora of surface modifications have been developed, all demonstrating an ability to modulate the activity of skeletal stromal cells to proliferate and then differentiate into bone synthesising osteoblasts.

In vitro assessments of osteogenic activity predominantly investigate how surface modification influences the biological responses of the skeletal stromal cells. Of these assays, cell survival and an ability to form attachments with serum proteins coating the implant surface are key questions. These will inform data arising from subsequent assays that indicate if the surfaces promote cell proliferation and cell differentiation towards an osteoblast phenotype capable of synthesising a mineralised matrix. However, the literature can produce contradictory studies, attributable to the numerous and varying *in vitro* methods of analyses used by researchers, with no “gold standard” for investigation definable. The problem is also that *in vitro* assays are prone to artefact and misinterpretation unless the underpinning biological basis for the assay, aligning to development of the osteoblast phenotype during the bone healing process, are fully appreciated.

This review aims to provide an evaluation of appropriate *in vitro* assays currently used for assessing the osseointegration process. In recognising that angiogenesis and macrophage function are also drivers for successful osseointegration, this review will additionally look at robust assays to assess the behaviour of these cells to implant surfaces. Overall, we aim to highlight some fundamental considerations to allow researchers to make more informed choices when designing experiments, interpreting the resulting data and evaluating data against that in the literature.

Bone biology during osseointegration

When we consider that osseointegration involves the production of new bone tissue that interfaces with an implant surface it is unsurprising that this process is not dissimilar to the process that occurs during fracture repair of bone. However, and more importantly, recognising the sequence of biological events associated with bone healing is vital for understanding methodological principles of *in vitro* assays used to evaluate osteogenic potential of an implant

surface and critical evaluation of results arising. The process of osseointegration can be briefly divided into four overlapping stages of: haematoma formation, inflammatory phase, reparative process and remodelling (Figure 1). Before implant placement, the relevant area of bone is drilled to a size that reduces any potential micromotion of the implant to a minimum and enhancing successful bone healing around the implant². The resultant process of bone healing is described in many other reviews^{3,4,5} and thus key cells and signalling factors are summarised in Figure 1. Depending on the surface properties and characteristics of the implant, a variety of proteins from the blood and surrounding interstitial tissue fluid are adsorbed onto the implant surfaces, that modulate binding of skeletal progenitor cells and pre-osteoblasts, through interactions with cell surface integrins⁶. Importantly, is the development of a haematoma which provides a stable framework for a wound healing site that facilitates a route of cell migration to the implant surface^{7,8}.

Other important cells are macrophages that are proposed to serve numerous roles including phagocytosis of foreign particles, bone debris and microbial contaminants^{9,10,11}. It is therefore unsurprisingly acknowledged that an “appropriate” initial inflammatory response is fundamental for initiating and propagating the subsequent reparative process leading to the deposition of woven bone^{3,4,5}. The local environment also contains a myriad of growth factors (Figure 1) that initiate the process of cell differentiation and tissue formation, during which angiogenesis is a prerequisite. Differentiating osteoblasts begin to secrete the osteoid matrix, which is rich in type I collagen (COL1) but also contains matrix proteins, such as matrix extracellular phosphoglycoprotein (MEPE) and osteopontin (OPN), that inhibit premature mineral deposition^{12,13}. Once the structural framework of the collagen matrix is laid down these inhibitors of mineralisation are removed by specific matrix metalloproteinases and the osteoblasts synthesise a new pool of non-collagenous proteins that guide and regulate the interfibrillar growth of hydroxyapatite crystals^{12,13}.

***In vitro* assessment for the assessment of osteoblast differentiation**

It is recognised that *in vivo* assessments will provide the necessary biological evaluation of medicinal products, conforming to ISO 10993 (comprehensively reviewed by Von Wilmosky *et al*¹⁴). However, a review of the literature clearly tells us that there is a great reliance on *in vitro* assessments, which are utilised to provide high throughput quantifiable data for measuring cell responses and understanding mechanistic signalling in a controlled culture environment; addressing the research question “is a surface capable of directly inducing an osseogenic response in bone marrow stromal cells (BMSCs).” Table 1 highlights the advantages of *in vitro* studies in providing an easier route to identifying those surface preparations that can provide good positive evidence for promoting osseointegration.

Nonetheless, the largest area for diversity in the research findings for *in vitro* studies is primarily linked to the starting population of cells used, which in turn can influence the results obtained for the fairly commonplace assays employed to quantify cell attachment, proliferation and osteoblast differentiation. An additional caveat to considering *in vitro* data is that bone formation *in vivo* involves paracrine regulation from other cells, including the various immune cells, osteoclasts and endothelial cells. Consequently, there is no guarantee that these observations translate to the *in vivo* scenario. This conclusion has been demonstrated in a multi-centre review which identified that there was no overall significant correlation between *in vitro* and *in vivo* outcomes in the assessment of biomaterials, and that drawing definitive conclusions between studies is thus incredibly difficult, which can be attributed to a number of credible compounding factors relevant to the assay protocol influencing result outcome¹⁵. This stated, *in vitro* and *in vivo* data, if performed and reviewed carefully to consider limitations and caveats, can be complementary and if demonstrating contradictory results, can be informative for the design of further studies to address the pertinent questions arising.

Considerations for cell source used in assays

A wide variety of cell types, all capable of differentiating into osteoblasts, are available for *in vitro* study. Table 2 highlights some of the more common cell types used to assess osteogenic potential and their advantages and disadvantages. The key information many studies aim to obtain is to provide a measure of osteogenic potency and efficacy. Potency can be defined as the ability to bring about a particular result, whilst efficacy relates to the measure of the intensity of the potent effect observed¹⁶. If studies are well controlled and starting conditions are standardised, the measure of potency and efficacy within a study is possible. However, efficacy is harder to compare when wishing to evaluate separate studies. This is because the various cell lines available for use vary greatly with respect to their phenotypic differentiation status, which will impact on cell proliferation and time required to achieve a fully differentiated osteoblast^{17,18}.

Cells derived from primary tissue source

Many studies use primary sources to derive BMSCs since they are considered to represent more closely the cells interacting with an implant surface *in vivo*. However, nomenclature for these cells provides one of the largest areas for ambiguities and in 2006, a working group of the International Society for Cellular Therapy (ISCT) recommended a new designation of multipotent mesenchymal stromal cells¹⁹ rather than mesenchymal stem cells (MSC). This is

because, and in the absence of definitive cell markers, a cell's "stemness" is difficult to evaluate in post-natal populations that are also likely to contain tissue specific progenitor cells and non-stem cells²⁰. This is supported by recent data to suggest that stromal cell populations isolated from bone marrow and then culture expanded contain variable concentrations of multi-potent cells; along with cells that are bi-potent for either osteogenic-adipogenic, osteogenic-chondrogenic, adipogenic-chondrogenic differentiation; uni-potent cells that represent lineage restrictive progenitor cells; and nulli-potent cells that fail to differentiate down one of the classical lineages, but are proposed to represent an immuno-supportive subtype^{17,18}. Consequently, a better recognised description for primary cells used in *in vitro* assay is to refer to these cells as stromal cells relevant to the tissue of primary origin (for example bone marrow stromal cells or calvarial stromal cells).

As a further consideration, variation in the proportions of these populations will influence the paracrine and autocrine signalling environment, which will in turn influence the quantitative data arising from *in vitro* assays. The profile of these stromal cells can also vary with increasing passage, where the more highly proliferative multi-potent cells assume dominance within the culture, although further continued passaging leads to deterioration of their reparative ability²¹. Other studies have suggested that osteogenic potential is lost through *in vitro* culture whereas adipogenic differentiation potential remains²². Moreover, when using human cells the heterogenic profile of BMSCs is subject to donor variability which can partially be attributed to age, race and gender, as well as systemic condition such as osteoporosis and type 2 diabetes, that can impair their regenerative capacity. Commercial sources for human BMSCs are available but patient information is limited and batch-to batch heterogeneity with respect to cell populations and manufacturing methods is a confounding factor²³. A further consideration is that within the bone marrow cavity these skeletal progenitor cells exist in either a perivascular or periosteal location and the two sets of cells differ markedly in the function they provide to bone remodelling and bone repair²⁴. Isolation of bone marrow may take variable proportion of perivascular and periosteal associated cells which can differ with respect to their proliferative and differentiation potential.

Sourcing primary skeletal stromal cells from rodent animals can, therefore, appear to provide several advantages in that age and systemic status of the donor animal can be controlled. However, inherent differences exist in the balance of immunological cells between human and rat or mice derived cells and considering the role of these cells in regulating bone turnover, it is not a surprise that differences exist between rodent and human bone marrow derived cells in their osteogenic potential²⁵. Indeed, it is noted that the differentiation of rat BMSCs into mature osteoblasts occurs between 7 and 14 days earlier compared with BMSCs from human origin, which can require up to 42

days culture to produce a dense mineralised matrix^{26,27}. Further, studies have indicated that mouse BMSCs isolated via plastic adherence contain higher levels of haemopoietic cells compared to human derived populations isolated via the same protocol^{28,29}. Numerous studies have also compared the osteogenic potential of stromal cells acquired from mandibular, where cells embryonically derive from the neural crest origin, and limb sites, where cells derive from the mesoderm. An overwhelming consensus has identified a greater osteogenic capacity for mandibular-derived stromal cells, compared to cells acquired from long bones, in species such as rats³⁰ and pigs³¹ and the iliac crests of humans³².

Osteoblast cell lines

Several commercial cell lines derived from osteosarcoma or spontaneously immortalised cells are available. These cells can offer advantages for providing an unlimited number of cells and represent a more homogenous cell population. However, one key consideration when using immortalised osteoblast cell lines is the numerous phenotypic differences compared to primary cell lines or osteoblasts. A comprehensive review by Czekanska *et al*³³ details how different immortalised and primary cell lines, cultured in identical media and conditions leads to differences in alkaline phosphatase (ALP) activity and mineral deposition within the synthesised matrix. This in turn, correlates with known variations in proliferation and osteoblastic marker expression. Significantly, studies have identified that SaOS-2, derived from a human osteosarcoma cell line, represents a mature osteoblast phenotype and thus do not mirror the whole range of osteoblast differentiation. MG-63 cells and SaOs-2, are reported to be p53 deficient³⁴. This arrests cells in the pre-osteoblast state, resulting in rapid cell division without contact inhibition, which is associated with an inability to express osteocalcin (OCN)³⁵, and associated inconsistencies in mineral deposition^{36,37,38}. MC3T3-E1, a clonal cell line derived from new-born mouse calvaria is a popular animal cell line, although immunological issues of interspecies difference described above are to be considered. As clonal cell lines MC3T3-E1 show good homogeneity, although cells at high passage show decreased proliferation, due to inconsistencies in cell cycling and evidence of replicative senescence, which influences post-proliferative stages associated with deposition of a mineralised matrix^{33,36}.

Seeding density

One recurring observation in reviewing the literature for *in vitro* assay protocols, is the variation in the seeding density of cells onto the implant substrate. Human and rodent derived cells are frequently seeded at densities as low as 5,000 cells/cm², to as high as 40,000 cells/cm², with rodent stromal cells generally seeded at the higher densities^{26,37,37,39}.

Seeding densities of osteoblastic cell lines range from between 10,000 cells/cm² up to 85,000/cm² ^{40,41,42}. A key consideration in deciding an appropriate seeding density relates to the widely accepted consensus of the reciprocal relationship between cell proliferation and differentiation; cells typically undergo an initial active proliferative phase which is then required to down-regulate prior to cell differentiation and osteoid production^{43,44}. This knowledge thus explains why BMSCs at a higher density in culture undergo osteogenic differentiation more readily than cells at a lower density⁴⁵. However, it is also noted that higher seeding densities of BMSCs can result in a bias towards adipogenic differentiation as opposed to osteogenic^{46,47}. Cell seeding density, reported for those cells in contact with the implant substrate, is therefore better optimised on an individual basis to ensure appropriate conditions that ultimately permit the researcher to confidently identify surface influences, initially examining active cell proliferation which is then observed to plateau prior to observation of differentiation markers towards an osteoblastic lineage.

Cell attachment and morphology

It is now well established that cell attachment to a matrix is necessary to promote cell survival and prevent anoikis (programmed cell death of anchorage-dependent cells following detachment from the surrounding extracellular matrix)^{48,49}, as well as dictating a cell's ability to proliferate and differentiate towards the osteoblast lineage. When exposed to human plasma, 48% of proteins adsorbing onto the titanium surfaces have been implicated in facilitating cellular adhesion⁵⁰. Formation of focal adhesion points between $\alpha v\beta_3$ and $\alpha_5\beta_1$ integrins on SaOS-2 osteoblast-like cells and RGD sequences within adherent proteins fibronectin or vitronectin coated surfaces have been shown to enhance cell proliferation compared to uncoated surfaces⁵¹. In addition, interactions of α_5 integrins with RGD peptide motifs has been associated with an upregulation of osteogenic differentiation of progenitor cells^{52,53}. However, for cells grown on plastic, pure titanium and calcium phosphate surfaces, differences in the profile of proteins adsorbed to the surface can lead to changes in integrin expression by cells⁵⁴. Surface roughness can also alter the profile of proteins absorbed, with rougher surfaces (Ra of 183.2 nm) adsorbing higher quantities of fibronectin and albumin compared to smooth machined surfaces (Ra of 53.5nm), which has in part been attributable to enhanced cell binding potential⁵⁵.

The formation of focal adhesion points can direct cell morphology. Cells with a branched cell morphology and a highly organised cytoskeleton, with defined focal adhesion points, correlate well with increased osteogenic potential⁵⁶. These changes in cell morphology and hence changes in the cytoskeletal proteins lead to the altered expression of several osteoblast genes, regulated via extracellular regulated kinase (ERK), focal adhesion kinase (FAK) and mitogen-

activated protein kinase (MAPK) signalling cascades^{57,58}. These studies, and many others also exemplify how the steric arrangement of cell surface focal adhesion points are important for directing cellular behaviour and this has formed a fundamental understanding for how surface topography can dictate cell behaviour (reviewed by Cutis and Wilkinson⁵⁹).

Media supplementation

A review of protocols used in the literature, indicates that around a third of studies chose to include osteogenic inducing factors within the culture media. There are many studies that do not supplement media with osteogenic induction factors, providing plentiful evidence that certain surface topographies are sufficiently capable of inducing osteogenic responses of BMSCs from humans and rodents⁶⁰⁻⁶³. For those that include osteogenic factors, the culture media is invariably supplemented with: dexamethasone, which up-regulates Runt-related transcription factor 2 (Runx2) expression; ascorbic acid, which stimulates collagen fibril synthesis; and β -glycerophosphate, which provides a source of phosphate (reviewed by Langenbach and Handschel⁶⁴). However, one research article of note is that of Sisti *et al*⁶⁵, where human BMSCs were cultured on various surfaces in both the absence and presence of 10nM dexamethasone. Interestingly, no differences in mRNA expression of ALP and OPN were observed for cells cultured on different surfaces in osteogenic-inducing medium, but significant differences for these markers were identified for cells cultured on the same surfaces in basal medium. Inclusion of osteogenic factors may therefore mask small to moderate osteogenic effects due to the surface, which are difficult to observe against the potent osteogenic influence of factors such as dexamethasone. Inappropriate supplementation can also produce false positive results. For example, treatment of cells, nominally incapable of osteogenic differentiation with ≥ 2 mM β -glycerophosphate can result in dystrophic mineralisation that stains positive for Alizarin Red or von Kossa^{60,64}. Further it should be remembered that commercial media preparation may already contain ascorbic acid and endogenous phosphate, reiterating the question is it necessary to add osteogenic supplementation?

Measuring early markers for osteoblast differentiation in vitro

Osteoblast differentiation is guided by a complex signalling pathway that requires the synthesis of transcription factors, growth factors, growth factor receptors and extracellular proteins that serve to regulate cell signalling and regulate (both through inhibition and promotion) mineral deposition. These all represent useful biomarkers for measuring osteogenic differentiation (Figure 2), recapitulating *in vivo* events that are measurable in differentiating cells within 2D culture environments⁶⁶. Analyses can be via measurement of gene expression or the synthesis of a protein.

What is often overlooked, however, is that the expression of many of the osteogenic genes is temporal; gene expression switches on at a specific maturational stage and is then required to be switched off again to allow differentiation to proceed (Figure 2). Analysis, therefore, only represents a “snapshot” of the cellular differentiation status at a specific moment in time. Consequently, it can be unclear whether analysis for the expression of a specific gene is measuring an increasing expression, at the peak of expression or when the gene is being regulated to switch off.

An ideal example to demonstrate this limitation in data interpretation is in the measurement of Runx2. Runx2 is widely regarded as a prerequisite transcription factor required for differentiation towards an osteoblastic phenotype^{67,68}. Runx2 expression is induced by DLX5, regarded as the master transcriptional regulator for osteogenesis and in turn Runx2 has been linked with promoting the expression of many of the bone matrix proteins such as COL1, OPN, bone sialoprotein (BSP) and OCN. However, studies have indicated that overexpression of Runx2 in differentiating osteoblasts severely reduces OCN expression by inhibiting osteoblast maturation^{67,68}. Consequently, Runx2 has been proposed roles in committing multipotent cells to pre-osteoblasts, but requires to be down-regulated in order to permit terminal differentiation to a mature osteoblast phenotype^{69,70}. Whilst levels of expression are variable depending upon a multitude of factors including cell type, initial seeding density, culture conditions and cell-material interactions, a limitation for many analyses is the difficulty in identifying the point in which Runx2 expression peaks before reducing expression to permit terminal differentiation, hence potentiating false results. Through monitoring of Runx2 expression at multiple daily time points it is possible to provide an indication for how rapidly an implant surface may induce osteoblast differentiation.

The use of Runx2 as a marker for osteoblast differentiation comes with additional forewarnings; the role of Runx2 as a master gene for indicating commitment of stromal cells to the osteoblast lineage is controversial. Whilst Runx2 expression has been indicated to inhibit adipogenic differentiation, its expression has been associated with chondrogenesis^{71,72}. Furthermore, it is reported that multipotent cells of human origin constitutively express Runx2⁷³. Indeed, it has been suggested that *in vitro* osteogenic differentiation of BMSCs with dexamethasone is more regulated by phosphorylation of the Runx2 protein, and not as a consequence of an increase in Runx2 expression, which is thought to remain the same⁷⁴. More specific to osteogenic differentiation is the expression for the gene encoding for osterix (*Sp7*), a transcription factor downstream of Runx2^{68,75}, which may be a better biomarker to determine pre-osteoblastic differentiation.

Measuring markers for mid to late osteogenic differentiation

In vitro studies also consider the expression of markers considered to be associated with either the development of the immature or mature osteoblast phenotype. One such marker is ALP, which is measured either at gene level or through colorimetric assays. ALP is an enzyme that plays a role in the hydrolysis and liberation of phosphate groups on proteins and in the hydrolysis of inhibitory pyrophosphates, providing a free source of phosphate ions for incorporation into the hydroxyapatite crystal lattice^{76,77}. These functions of ALP mean that the enzyme is expressed in high levels in immature osteoblasts and is widely used as an indicator of early osteogenic differentiation^{78,79,80}. However, it is also important to consider that ALP isoforms are also present in multipotents⁸¹ and pluripotent cells⁸² and is down regulated when cells enter the M phase during the cell cycle⁸³.

Another regular marker for osteogenic differentiation is OPN (also known as secreted phosphoprotein, SPP1). Within the bone healing process OPN is synthesised by pre-osteoblasts and immature osteoblasts, but it is also highly synthesised by macrophages⁹. OPN binds to newly exposed bone surfaces to become incorporated in cement lines delineating new and old bone surfaces⁹. It binds to debris bone pieces and serves as an opsonin for clearance by macrophages⁹. Whilst OPN appears to be one of the earliest secreted proteins, it has been identified as a negative regulator for the proliferation and differentiation of MC3T3-E1 cells, where its overexpression has been shown to inhibit responsiveness to bone morphogenic protein-2 (BMP-2)⁸⁴. OPN has been shown to bind to specific faces of the hydroxyapatite crystal where it inhibits mineral crystal growth^{85,86}. Thus, it is essential to demonstrate down-regulation of OPN to demonstrate full differentiation to a mature osteoblast phenotype.

Considering that osteogenic markers may not be continually expressed at high levels for the duration of the osteogenic differentiation, better studies provide analysis for multiple osteogenic markers to identify early, mid and late stage of differentiation, at multiple time points in order to help maximise the possibility of identifying the peak expression. Additional markers of differentiation towards osteoblasts that can additionally be assessed to include, but not limited to, COL1, BSP and OCN³³. However, possibly the clearest marker for the formation of a mature osteoblast is its ability to deposit a mineralised matrix which can be measured using protocols for staining of calcium by Alizarin red and its release following subsequent dissolution of bone mineral nodules with acetic acid⁸⁷. The deposition of a biological apatite, and not monophasic forms of calcium phosphate should ideally be confirmed, which is possible using techniques such as FTIR (Fourier Transform InfraRed spectroscopy)⁸⁸.

***In vitro* assessment of macrophage activity**

Macrophages play important roles in the clearance of cell debris, bacteria and other small particulates such as small bone debris at the implant site⁹. They also play important roles in regulating osteoblast activity via the expression of pro- and anti-inflammatory cytokines⁸⁹. However, when assessing the osseointegration potential of a titanium surface, neglecting the role of the immune cells is a frequent omission to many of the published studies. Macrophages are now recognised to broadly exist in two distinct polarized subsets; M1 which is ascribed roles in destroying pathogens and propagation of the pro-inflammatory response, and M2 which is attributed roles in tissue repair, angiogenesis and re-establishment of homeostasis^{89,90} (Figure 3). A transition from the M1 phenotype to the M2 phenotype is seen as prerequisite for good bone healing^{89,90}, where prolonged presence of M1 macrophages has potential to extend inflammation and thus delay reparative responses^{89,90}. The polarisation to M1 or M2 can readily be distinguished. M1 characteristically release pro-inflammatory cytokines interleukin 1 β (IL-1 β), IL-6, IL-12, IL-23, TNF- α along with iNOS and carry cell surface markers CD86 and CCR7^{89,90}. M2 polarised macrophages are characterised by cell surface markers CD163 and CD206 and the production of anti-inflammatory factors such as IL-10, transforming growth factor- β (TGF- β), BMP-2, vascular endothelial growth factor (VEGF) and Arginase-1^{89,90}, some of which are notable for their role in promoting osteoblast differentiation and bone healing. *In vitro* studies investigating macrophage polarisation may use monocytic cells isolated from blood⁹¹ or bone marrow⁹² which offer the advantage that they derive from a human source, but the isolated cells will be heterogeneous with respect to the proportions of M1 and M2 phenotypes, amongst other monocytic cells. Studies have also used commercial transformed lines such as the well established RAW 264.7 cells or the human derived THP-1 monocytic cells, which may be easier to culture, but come with the cautions of using immortalised cell lines.

The important role macrophage polarization plays in influencing the osseointegration process and osteoblast function has been used to explain differences witnessed in the osteogenic potential of titanium surfaces seen in *in vitro* and *in vivo* studies. Using a range of established *in vitro* osteogenic assays, a series of publications have studied how the width of nanotubes fabricated onto titanium surfaces influences the osteogenic process; indicating that those etched with wider nano-tubes demonstrated a greater osteogenic response^{91,92}. However, *in vivo* studies using the same etching patterns suggested that those surfaces etched with the smaller diameter nano-tubes promoted bone formation, which was associated with less inflammation^{91,92,93}. This dichotomy was addressed following analysis of recovered conditioned media from macrophage cells cultured on the titanium surfaces, which suggested that those surfaces promoting M2 polarisation also promoted osteoblast differentiation of BMSCs^{92,93} and concurs with reports

that M2s secrete BMP-2 and TGF- β ^{94,95}. Additionally, M2 polarisation has also been shown to promote *in vitro* angiogenesis of human umbilical vein endothelial cells (HUVECs), attributable to VEGF secretion⁹⁶.

Studies have additionally reported that hydrophobic titanium surfaces result in a diminution of M2 phenotype as judged by increases in pro-inflammatory gene expression^{97,98} and it has been hypothesised that this surface feature has the greatest effect in determining macrophage fate⁹⁹. The ability of hydrophilic and rough surfaces to promote M2 polarisation has been hypothesised to be attributed to enhanced protein adsorption and maintenance of protein conformation, thereby providing attachment for the cells. Attachment of macrophages to a protein adsorbed surface allows for cell spreading and induces changes in cell shape facilitated by changes in F-actin content and cell surface adhesion proteins¹⁰⁰. The importance of signalling via the actin cytoskeleton has been demonstrated using cytochalasin B, which binds to the ends of extending actin filaments and inhibits cytoskeletal-dependant shape changes¹⁰¹. High concentrations of this inhibitor produced rounded macrophages with reduced BMP-2 secretion. Additionally, the cytoskeleton influences cell contact and adhesion sites, where the contribution of β 1 and to a lesser extent β 3 integrins in the process of M2 polarisation has been indicated using blocking antibodies¹⁰².

Assessment of angiogenesis as part of the osseointegration process

The requirement for new blood vessel formation in promoting successful bone repair and osseointegration of an implant is well acknowledged. Signalling factors produced by endothelial cells bring reparative skeletal stromal cells to the wound site^{103,104} and regulate maturation into osteoblasts¹⁰⁵. For the few studies investigating *in vitro* responses of endothelial cells to titanium surfaces, HUVECs are commonly used as a highly characterised and commercially available cell line. Simple protocol procedures have been successfully employed to isolate HUVECs from an umbilical tissue source^{106,107,108} and buffy coats from blood¹⁰⁹. These populations are inevitably heterogeneous, containing variable numbers of endothelial precursor cells, which further divide into colony-forming cells or late outgrowth cells, which differ considerably with respect to their morphology and proliferative potential¹¹⁰.

In vivo, angiogenesis involves either endothelial sprouting or intussusceptive angiogenesis (forming new vessels through the splitting of one vessel) and smooth muscle cells and pericytes line the endothelial vessels¹¹⁰. *In vitro* angiogenic assays do not usually consider these additional mural cells, and thus established assay protocols are limited to investigating early angiogenic responses. This can include assessment of cell proliferation by either direct cell counts¹⁰⁷, or via assays that correlate with cell metabolic reductase activity such as the MTT assay^{106,110} or the Alamar blue

assay¹⁰⁶. The influence of surfaces on cell survival can also be readily assessed using apoptosis assays or the detection of other pro-apoptosis factors such as caspase-3, Bax and Fas ligand¹¹¹ or the secretion of angiopoietin-1 (Ang-1), VEGF, platelet derive growth factor (PDGF) and fibroblast growth factor-2 (FGF-2)^{112,113,114}; the latter grouping representing factors that also positively promote the proliferation and differentiation of progenitor cells to osteoblasts^{103,104}. Although limited in number, studies have suggested that greater numbers of endothelial cells adhere to smooth surfaces compared to rough surfaces¹⁰⁹. Conversely, functionalisation of titanium with TiO₂ nanowires has been shown to enhance secretion of VEGF and increase mRNA expression of VEGF, FGF-2, PDGF and fibroblast growth factor receptor 2 (FGFR2) in HUVECs when compared to un-functionalised surfaces¹⁰⁸.

Monitoring of cell migration using wound scratch assay on titanium surfaces has been shown to be more problematic, primarily due to difficulties in viewing the cells on an opaque surface which necessitates fluorescence staining of cells in order to assess wound closure at fixed time points^{106,108}. Likewise, *in vitro* assessments for the ability of titanium surfaces to support tubular network formation and terminal endothelial differentiation have proved difficult. This is because *in vitro* tubulogenesis assays require endothelial cells to be incorporated within or on top of an extracellular matrix gels and coating surfaces with these gels usually abrogates the assessment of geometric surface modification or surface functionalisation.

Concluding remarks

Currently, there is no consensus for identifying a standardised series of *in vitro* assays able to quantitatively assess the impact of an implant modification for promotion of the bone healing process. This review has described several protocols for which there are strengths and limitations and consequently demonstrates that reporting of a robust methodology to produce confidence in the data generated is paramount. As the provider of biological information, the selection of an appropriate cell line needs careful consideration and results need to be interpreted around cellular characteristics and the heterogeneous potential of the population. The addition of osteogenic factors can additionally create super-physiological osteogenic induction, which can mask the true osteoinductive potential of the implant surface. The better *in vitro* assays aim to map the changing differentiation status of the skeletal stromal cells through to a mature osteoblast, where in order to obtain sufficient discrimination in analysis, assays monitor several osteogenic markers, acquiring a series of data points over a time course relevant to their temporal expression. The research aim for many studies can thus identify whether a surface modification is able to accelerate the

osseointegration process. However, this review has also highlighted how osteoblast differentiation is influenced by the paracrine signalling from other cells such as macrophages. Indeed, additional *in vitro* studies that assess the influence of these cells have been demonstrated to provide valuable information that allows better interpretation of subsequent *in vivo* data.

Ultimately, *in vitro* analyses do provide important research tools for rapid and relatively inexpensive screening of the many different implant surface designs now possible. They can provide data which provide the scientific justification to support selection of those surfaces to be taken through for *in vivo* assessments. In this regard, *in vitro* assays contribute to the ethical reduction of animal experimentation, although they cannot fully replace *in vivo* investigations. However, if reductionist aims are to be successful, it is important that the strengths and limitations of *in vitro* assays should be recognised to provide a critical assessment and prevent the selection of false positive surfaces.

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Table 1: Comparison of factors for consideration when selecting *in vitro* or *in vivo* methodologies.

Factor for consideration	<i>In vitro</i>	<i>In vivo</i>
Cost	Relatively cheap – Utilises various cells, many of which can be culture expanded to enhance cost effectiveness.	Expensive – Costs of animal purchase, care, housing, etc. in addition to costs for post-analysis
Technical difficulty	Technically simple	Technically challenging – Requires animal handling training, knowledge of surgical technique, etc.
Licensing requirements	No licence required	Requires ethical licence
Data acquired and evaluation	Usually limited to assessing effects on one cell type per assay (unless using co-culture system)	Holistic evaluation based on many cell types in a dynamic, living system
Ethical considerations	Limited to source of cells and reagents	Multiple ethical considerations
Technicalities of model systems	Absence of bio-kinetics may cause results not to be extrapolated to <i>in vivo</i> Cells can undergo changes throughout culture (cell instability)	Differences in animal species to humans may cause results not to be extrapolated clinically Different animal species have different bone growth and maturation rates compared to humans

Table 2: Advantages and disadvantages of commonly used *in vitro* cell models.

Cell type	Advantages	Disadvantages
Primary human BMSCs	Relevant for clinical translation Safe from malignant transformation	Limited replicative lifespan in culture Phenotypic alterations with prolonged culture Varying phenotypic characteristics based on tissue source Donor variability
Primary human osteoblasts	Ability to study bone matrix deposition and mineralisation independently of other cells	Limited supply Long isolation procedure
Primary rodent BMSCs	High differentiation potential towards osteoblasts	Reduced differentiation capacity throughout culturing Interspecies phenotypic differences Higher rate of differentiation compared to human MSCs
Primary rodent osteoblasts	Comparable developmental sequence to primary human osteoblasts More rapid isolation compared to primary human osteoblasts	Variations in differentiation potential between species Slow proliferation immediately after isolation
MG-63	Unlimited cell number	Uncontrolled proliferation Lack expression of differentiated osteoblasts Poor mineralisation potential P53 deficient
SaOS-2	Reproducible synthesis of mineralised matrix Temporal expression of mature osteocytic genes	Lack growth contact inhibition (not restricted to monolayer growth) P53 deficient
7F2	Indefinite growth potential Homogeneous population	Intermediate differentiation capacity between adipocytes and osteoblasts
MC3T3-E1	High differentiation capacity towards osteoblasts Undergo sequential steps of differentiation	Reduced mineralisation capacity throughout culturing Sub-clone variations
U2-OS	Responsive to BMP-2 Support outgrowth of haematopoietic endothelial cells Possess functional p53 tumour suppressor gene Narrower range of chromosomal aneusomy compared to p53 deficient cells	Aneuploidy
ROS 17/2.8	Mature osteoblast-like cells, thus more reproducible osteogenic response.	Differential potential limited to late stages
UMR-106	ALP expression indicative of late stage osteoblasts	Exist as two sub-clones (differentiated by from presence of calcitonin receptors)
MBA-15	Possess <i>in vitro</i> and <i>in vivo</i> osteogenic differentiation potential	Differentiation potential limited to early stage only

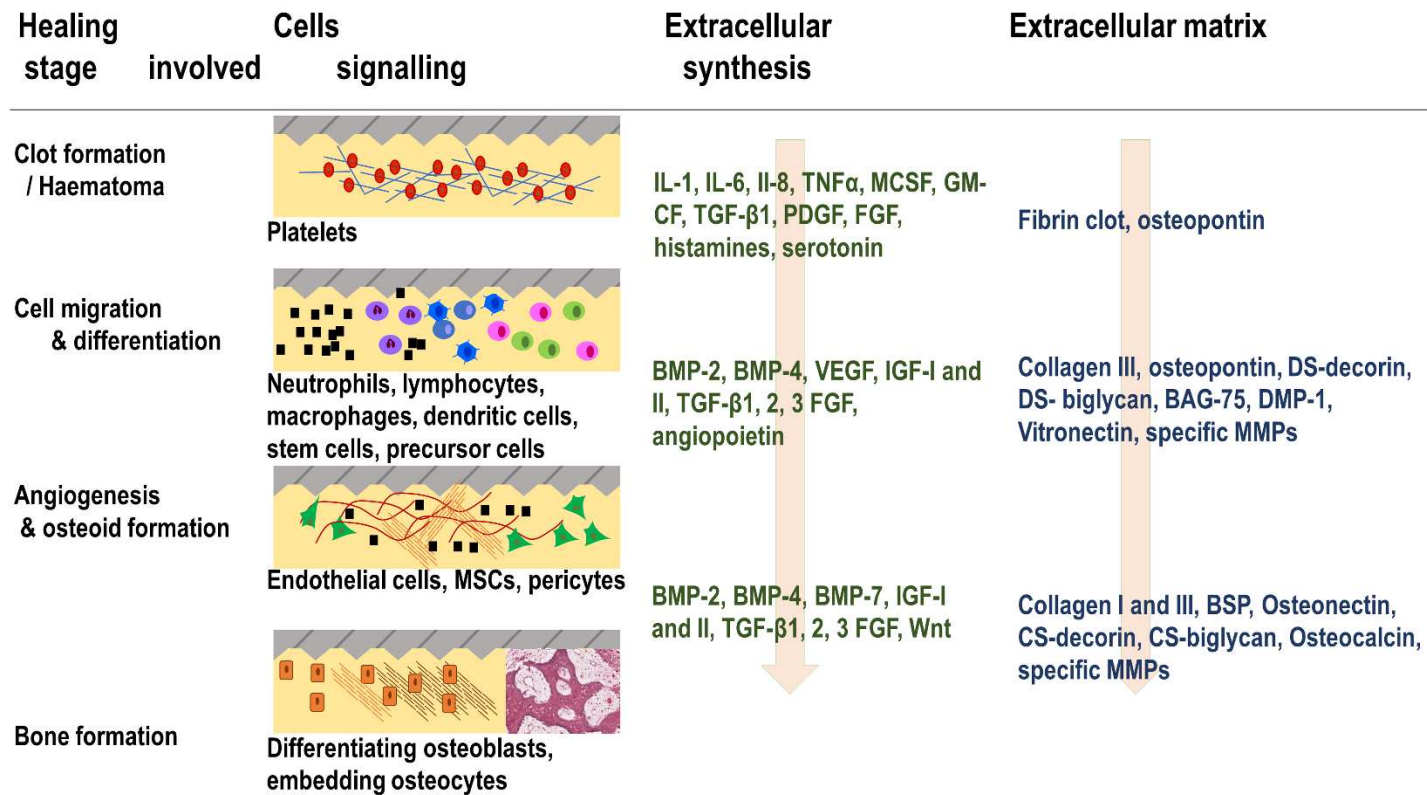


Figure 1: The four continuous and overlapping stages of bone healing. Each stage involves the activities of different cell populations, responsible for the synthesis of an extracellular matrix which acts as a scaffold and reservoir for known growth factors and cytokines. The signalling activity of these proteins encourages the recruitment of other cells, including immune, endothelial and stromal cells which bring about remodelling of the healing tissue and a change in the signalling environment. This allows for the removal of foreign particles, angiogenesis and ultimately the deposition of a bone matrix. Key cells, signalling factors and the extracellular matrix they interact with are shown.

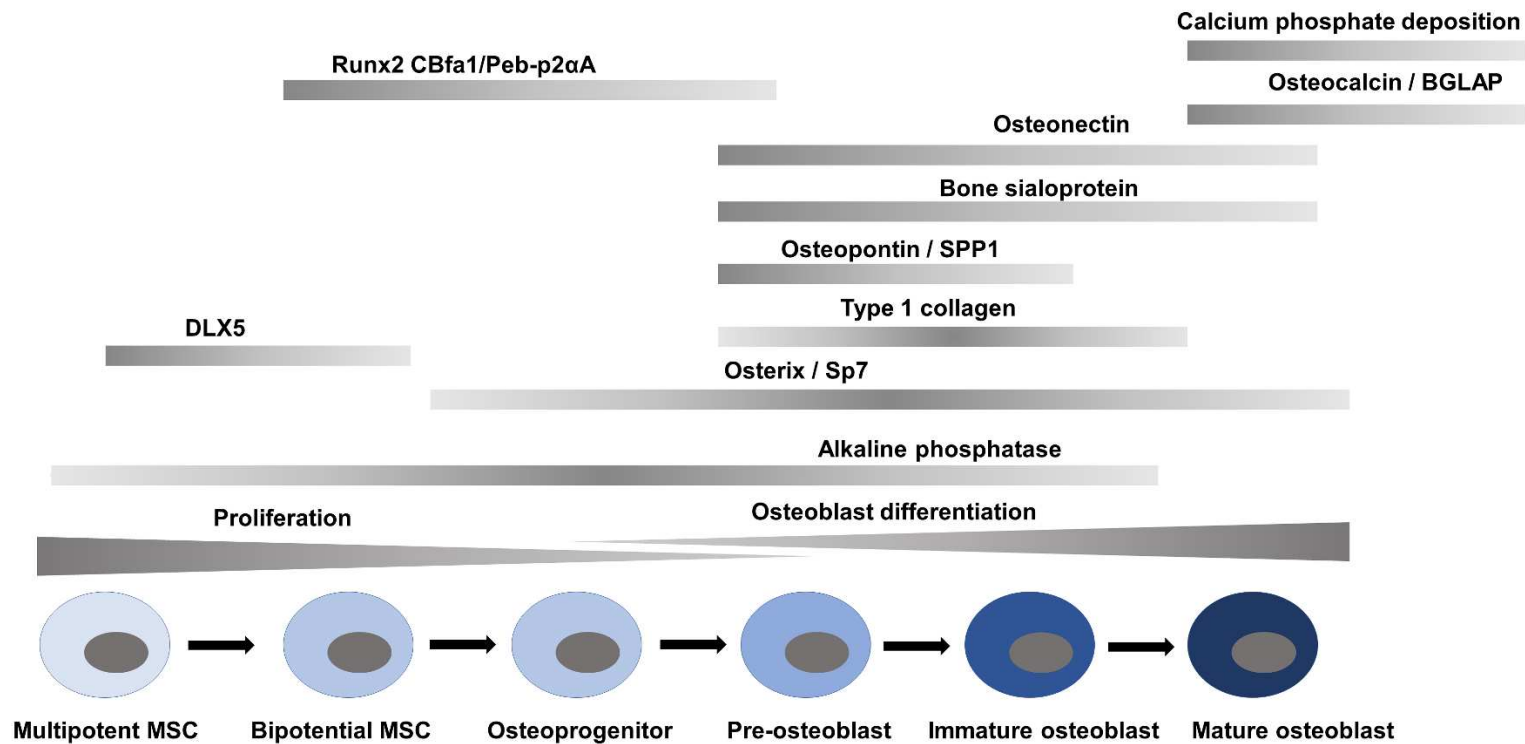


Figure 2: The temporal appearance of bone osteogenic markers produced during the culture of skeletally-derived stromal cells. Dependent upon the starting seeding density, cells initially enter a phase of proliferation which then down-regulates before osteoprogenitor cells enter terminal differentiation down the osteoblast lineage. It is important to recognise that some of the osteogenic markers are now known not to span the full range of osteoblast differentiation status. Dependent upon the cell source used in the assay, cell populations will be variably heterogeneous for the different osteoblast phenotypes described.

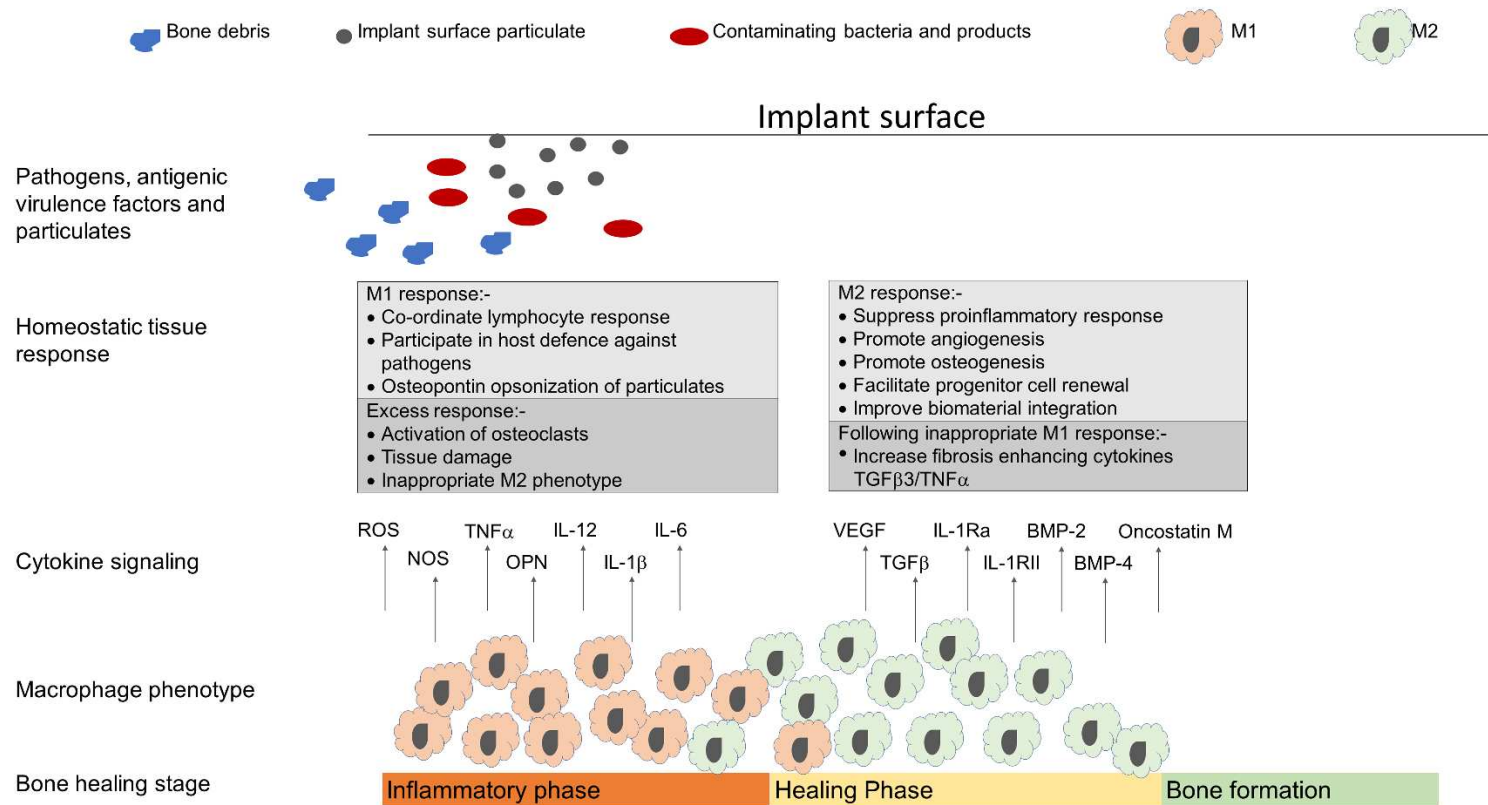


Figure 3: The role of macrophages in the bone healing process. During early wound healing the M1 phenotype is proposed to predominate which is pro-inflammatory in nature and performs roles in the removal of bacteria, bacterial antigens and particulate matter and ions released from an implant surface. As bone healing progresses the macrophage phenotype changes to the anti-inflammatory M2. Prolonged presence of M1 has been associated with delayed healing and tissue fibrosis. Biomaterials that promote M2 phenotype have been associated with improved integration due to improved tissue healing. Figure represents summary of information from published reviews^{88,89}.