

**EPIGENETIC APPROACHES: THE EMERGING ROLE OF  
HISTONE DEACETYLASE INHIBITORS (HDACis) IN  
PROMOTING DENTAL PULP CELL REPAIR MECHANISMS  
IN VITRO**

by

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## Abstract

Despite recent improvements in the clinical outcomes of vital pulp treatment, existing approaches remain non-specific and unpredictable. Developing biologically-based therapies that promote pulp regeneration is critical. Epigenetic modifications of DNA and histones control cellular processes, including proliferation, mineralisation and stem cell fate, and therefore offer exciting therapeutic opportunities. Chromatin acetylation can be altered pharmacologically using histone-deacetylase-inhibitors (HDACis), which relax its structure and modulate transcription. This project investigated regenerative-associated HDACi effects *in vitro* on a cell-line and primary dental-pulp-cells (DPCs), using proliferation, viability, mineralisation, cell-migration, enzyme activity, high-throughput gene/protein expression and pathway analyses. HDACis increased DPC differentiation and mineralisation-associated gene/protein expression at concentrations, which did not reduce viability. Primary DPC mineralisation was promoted without altering cell viability/apoptosis, indicating a resistance to HDACi-mediated toxicity compared with cell-lines. HDACi-induced DPC reparative processes were mediated by matrix metalloproteinase (MMP) expression and activity. MMP-13 inhibition further increased mineralisation-associated events, but decreased cell-migration indicating a novel role for MMP-13 in pulpal repair. HDACi solutions released a range of previously characterised and unreported bioactive dentine matrix components, which may further supplement regenerative capability *in vivo*. Results demonstrate that HDACi directly stimulate DPC repair-associated events, highlighting their potential for augmenting dental materials or pulp-engineering scaffolds for regenerative endodontics.

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## List of Abbreviations

<b>AAE</b>	American Association of Endodontists
<b>ANOVA</b>	Analysis of variance
<b>Ca(OH)<sub>2</sub></b>	Calcium hydroxide
<b>DMC</b>	Dentine matrix component
<b>DMP-1</b>	Dentine matrix protein-1
<b>DNA</b>	Deoxyribonucleic acid
<b>DNMT</b>	DNA methyltransferase
<b>DPC</b>	Dental pulp cell
<b>DPSC</b>	Dental pulp stem cell
<b>DSPP</b>	Dentin sialophosphoprotein
<b>ESC</b>	Embryonic stem cell
<b>GF</b>	Growth factor
<b>HAT</b>	Histone acetyl transferase
<b>HDAC</b>	Histone deacetylase
<b>HDACi</b>	Histone deacetylase inhibitor
<b>iPSCs</b>	Induced pluripotent stem cells
<b>lncRNA</b>	Long non-coding RNA
<b>miRNA</b>	MicroRNA
<b>MDPC-23</b>	Murine odontoblast-like cells
<b>MMP</b>	Matrix metalloproteinase
<b>MTA</b>	Mineral trioxide aggregate
<b>ncRNA</b>	Non-coding RNA
<b>RNA</b>	Ribonucleic acid
<b>SAHA</b>	Suberoylanilide hydroxamic acid
<b>SC</b>	Stem cell
<b>SCAP</b>	Stem cells from apical papilla
<b>SHED</b>	Stem cells from human exfoliated deciduous teeth
<b>siRNA</b>	Small interfering RNA

**sncRNA**

Short non-coding RNA

**TSA**

Trichostatin A

**VPA**

Valproic acid

## **CHAPTER 1**

### **GENERAL INTRODUCTION**

## 1.1 Dental pulp infection, injury and repair

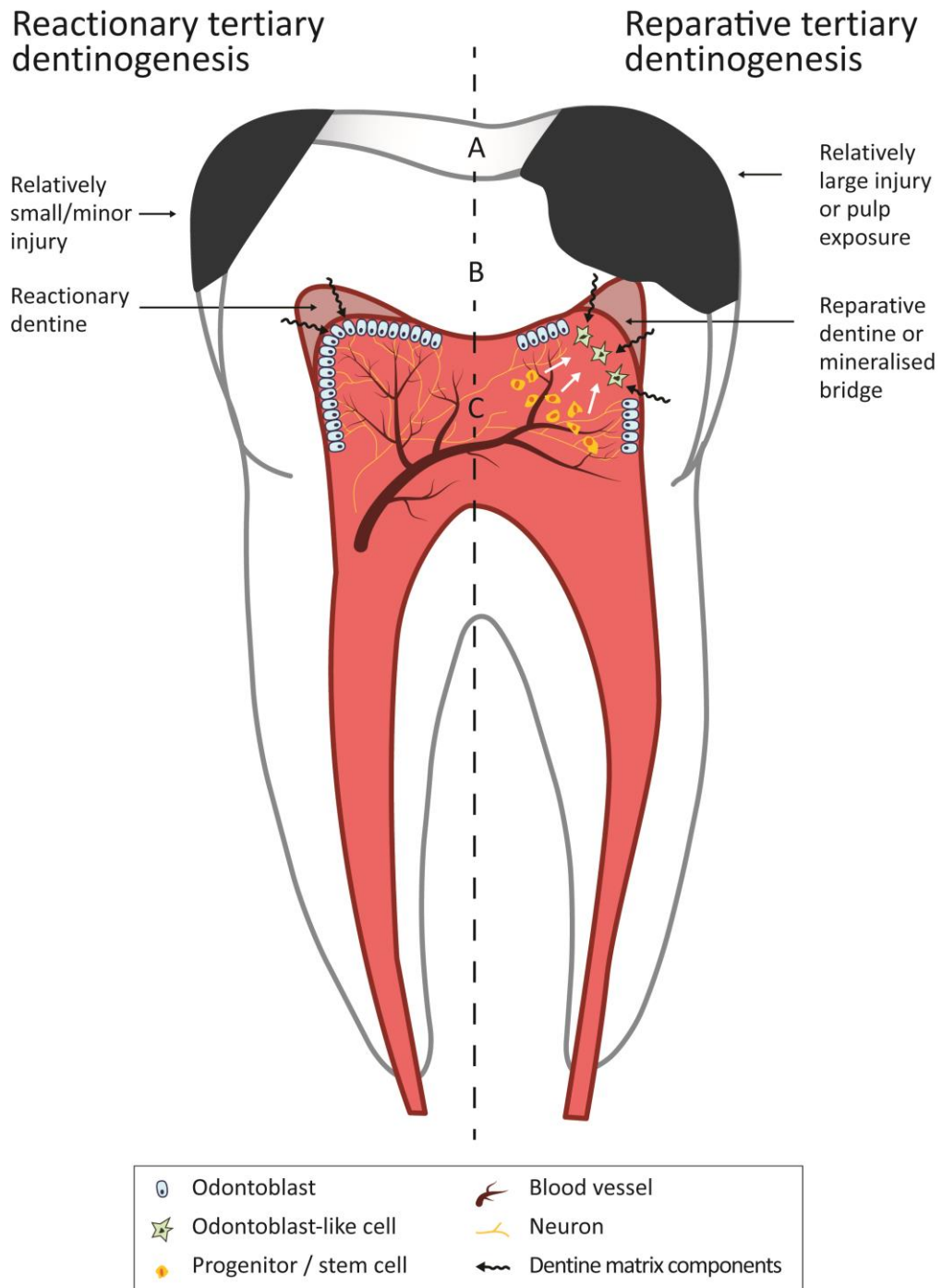
The preservation of healthy dental pulp tissue and subsequent prevention of apical disease, form the biological basis of operative dentistry. In a healthy tooth, the pulp is naturally protected by a mineralised outer shell of enamel and dentine, while also possessing a range of cellular defence strategies designed to protect against injury. The pulp can be challenged by stimuli including caries, trauma and dental restorative dental procedures, all of which provoke inflammatory responses in the pulp, the nature and extent of which reflects the severity of the challenge (Mjör & Tronstad, 1972). Microbial infection in caries lesions or 'leakage' around dental restorations provides the principal pulpal challenge, as bacterial products diffusing through the dentinal tubules induce inflammation even when the caries process or the restoration has not yet reached the pulp (Warfvinge & Bergenholtz, 1986). A range of pulp cells react immunologically to the microbes including initial pathogen recognition by odontoblasts and later fibroblasts, stem cells and immune cells; thereafter, a complex series of antibacterial, immune and inflammatory responses are activated (Farges *et al.*, 2009 & 2015; Soden *et al.*, 2009). As the carious process advances towards the pulp, the inflammatory process intensifies and the nature of the bacterial microflora change from aerobic to predominately anaerobic in deep carious lesions (Nadkarni *et al.*, 2004; Chhour *et al.*, 2005). If the caries progresses untreated, the microbial biofilm will advance and the associated bacteria will invade the tissue. This aggressive bacterial challenge invariably leads to irreversible pulpitis, pulp necrosis and subsequent apical periodontitis (Reeves & Stanley, 1966). Over time the bacterial flora in the diseased pulp and the subsequent necrotic root canal system change from comprising principally facultative anaerobic bacteria to more gram-negative, obligate anaerobic bacteria (Fabricius *et al.*, 1982; Rôças *et al.*, 2015). Pulp

necrosis will necessitate remedial dental treatment, such as tooth extraction or root canal treatment.

Pulp tissue, however, has an innate ability to heal if the challenge is removed and the tooth is suitably restored (Mjör & Tronstad, 1974). It appears that a controlled level of inflammation is critical, at least initially, to drive the repair process (Cooper *et al.*, 2010). If possible, biologically-based treatments, such as pulp capping and pulpotomy, aiming to maintain an intact pulp are preferable to root canal treatment, which is more costly, complex, time-consuming and destructive of sound tooth tissue (Stanley, 1989; Reeh *et al.*, 1989). Clinically, maintaining pulp vitality is of particular importance if the tooth is immature and root formation is incomplete.

### **1.1.1 Dentine and pulp**

The dental pulp occupies the centre of the tooth (Fig 1.1) and is responsible for forming dentine developmentally and throughout the life of the tooth. Odontoblasts are located at the periphery of the pulp, forming an interface with the dentine, which results in an anatomically and functionally linked tissue, traditionally referred to as the dentine-pulp or pulpo-dentine complex (Pashley, 1996). The odontoblast is a secretory cell responsible for the formation of primary dentine during tooth development and later for both the slower production of secondary dentine throughout the lifetime of the tooth and for tertiary dentine production when challenged (Simon *et al.*, 2009). Additionally, odontoblasts are involved in sensory stimulus transmission from the dentine and play an immunocompetent role in cellular defence (Couve *et al.*, 2013). A thin enamel layer generally protects the underlying pulp-dentine complex, which if its integrity is compromised by microbes, wear or



**Figure 1.1 Schematic of the processes of tertiary dentin formation.** Reactionary and reparative dentinogenesis processes differ in the source of the secreting cell. Reactionary dentine is formed by the existing primary odontoblast with a mild stimulus (early stage carious disease) stimulating upregulation of existing odontoblast activity. During the reactionary dentinogenesis process the odontoblasts recognise the bacterial products and released dentine matrix components (DMCs) diffusing through the dentine tubules, which increases cellular activity. Reparative dentine formation involves a more complex sequence of events in which a severe stimulus (increased carious involvement of dentine) causes death of the primary odontoblasts, which are subsequently replaced following differentiation of progenitor or stem cells into odontoblast-like cells under the regulation of bioactive molecules (including DMCs). As the nature of the cellular response is likely to be dependent upon the pulp environment, the mineralised tissue deposited at the pupal wound site will likely display a spectrum of dysplasia. **A** Enamel, **B** Dentine, **C** Pulp.

operative procedures, places the pulp at risk of harm. The pulp tissue responds to these challenges by localised inflammation and the production of tertiary dentine, which forms beneath the area of challenge (Lesot *et al.*, 1994; Smith, 2002). There are two types of tertiary dentine formed (Fig 1.1) depending on the severity of the irritating stimulus; mild irritation induces an up-regulation of existing odontoblast activity to form reactionary dentine, while stronger stimuli result in odontoblast death and the recruitment of dental pulp stem/progenitor cells, which differentiate into odontoblast-like cells to form reparative dentine (Lesot *et al.*, 1994). This cyto-differentiation and reparative dentine formation is regulated by bioactive molecules, including bone morphogenic proteins and growth factors (GF), which are 'fossilized' in the dentine matrix (Cassidy *et al.*, 1997; Smith, 2003; Grando Mattuella *et al.*, 2007) prior to being released by caries, trauma or dental materials (Graham *et al.*, 2006; Tomson *et al.*, 2007).

Clinically, the reparative dentine forms a mineralised bridge, which helps protect the pulp tissue from further insult (Glass & Zander, 1949; Nyborg, 1955). Reparative dentine formation evidently involves a complex series of biological events, which are not fully elucidated, however, bioactive molecules released from the dentine or pulp matrix and from altered pulp cell transcription following injury to the pulp-dentine complex are central to the process (Rutherford *et al.*, 1993; Nakashima, 1994; Cassidy *et al.*, 1997; Smith & Lesot 2001; Iohara *et al.*, 2004). From a histological viewpoint, pulp exposure healing should be described as the formation of a continuous hard tissue barrier over the exposure and a residual pulp, free of inflammation (Schröder, 1973). However, treatment outcomes for pulp capping can only be evaluated clinically and radiographically (Woehrlen, 1977; Fuks *et al.*, 1982).

### **1.1.2 Dental pulp and progenitor cells**

During reparative dentinogenesis, the recruitment of a progenitor cell population to the injury site, prior to their expansion and differentiation into odontoblast-like cells, is central to the ability of the dental pulp to repair (Smith *et al.*, 2016). However, the location and nature of the progenitor cells are the subject of debate, and they have been attributed to stem cell (SC) populations within the pulp (Smith & Lesot, 2001), SCs migrating from outside the tooth (Feng *et al.*, 2011; Frozoni *et al.*, 2012) and also undifferentiated mesenchymal cells from cell-rich and central pulp perivascular regions, namely pericytes (Fitzgerald *et al.*, 1990; Machado *et al.*, 2015). At present, there is a lack of consensus regarding the progenitor population responsible for reparative dentine formation, although surface marker analysis generally confirms a mesenchymal origin (Simon & Smith, 2014). Within the pulp core, there are two characterised post-natal SC populations, dental pulp SCs (DPSCs) and SCs from human exfoliated deciduous teeth (SHEDs), which reportedly account for 1-5% of total permanent and 2-9% deciduous pulp cell populations, respectively (Gronthos *et al.*, 2000; Miura *et al.*, 2003; Coppe *et al.*, 2009). DPSCs, like other SC populations (Crisan *et al.*, 2008), reportedly reside in perivascular areas, potentially to enable mobilisation to the wound site (Shi & Gronthos, 2003; Casagrande *et al.*, 2011). Critically, the influence of infection and pulp inflammation will alter the cellular response and quality of the mineral produced (Ricucci *et al.*, 2014) and as a result, the interaction of inflammation, materials and SCs post-injury needs to be fully understood if the development of innovative regenerative solutions are to be realised (Cooper *et al.*, 2010).



### **1.1.3 Regenerative endodontics and treatment of the exposed pulp**

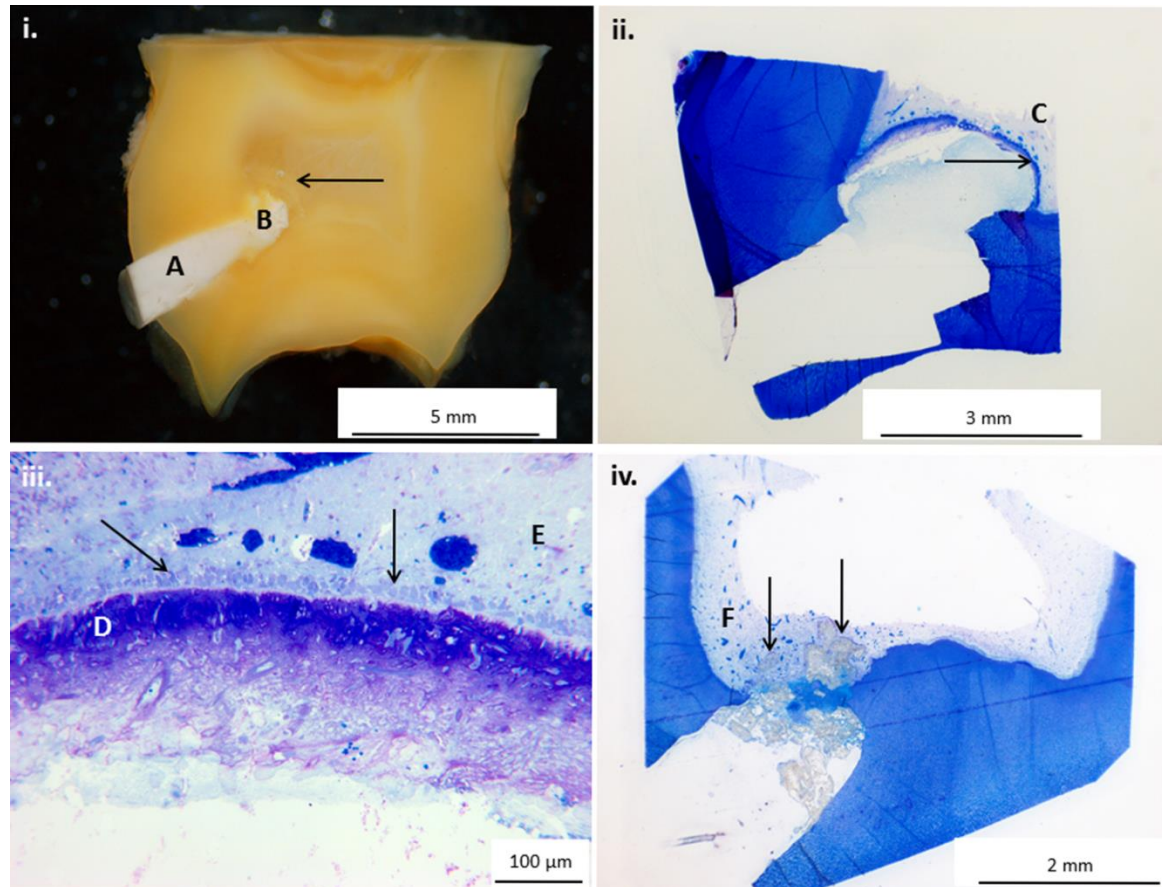
There is a history of pulp preservation within restorative dentistry, which can be traced back to the 18th century (Glass & Zander, 1949), when gold was inserted over an exposed pulp to promote healing. During the course of the last century, the popularity of these pulpal regenerative techniques has fluctuated, with Rebel seminally stating that 'the exposed pulp is a doomed organ' (Rebel, 1922). Unfortunately, this message has endured despite decades of research on the subject indicating results to the contrary (Nyborg, 1955 & 1958; Haskell *et al.*, 1978; Baume & Holz, 1981; Schröder *et al.*, 1985; Farsi *et al.*, 2006; Bogen *et al.*, 2008; Hilton *et al.*, 2013). The reluctance of clinicians to accept vital pulp treatment procedures may be due to often unpredictable results when compared with more conventional forms of treatment, such as root canal therapy (Strindberg, 1956; Haskel *et al.*, 1978; Barthel *et al.*, 2000), which can be attributed at least in part, to an incomplete understanding of pulp biology and the interaction with dental restorative materials. Recently, a better understanding of pulp defence and repair (Smith, 2002), the advent of new improved dental materials (Moghaddame-Jafari *et al.*, 2005; Nair *et al.*, 2008) and the promotion of regenerative endodontic therapies (Smith, 2002; Murray *et al.*, 2007; Nair *et al.*, 2008) has stimulated a new wave of research and treatment protocols in this area. As a result, vital pulp treatment procedures, within the last ten years, have demonstrated a predictable outcome approaching or even exceeding that of conventional root canal treatment (Qudeimat *et al.*, 2007; Bogen *et al.*, 2008; Hilton *et al.*, 2013).

In essence, pulp capping procedures can be viewed as being akin to natural bone healing processes with restorative treatments facilitating pulp-dentine GF release (Smith *et al.*, 2016), which stimulates the migration and differentiation of progenitor cell populations

within the extracellular pulp matrix to enable a regenerative response at the injury site (Schneider *et al.*, 2014; Smith *et al.*, 2016). Perhaps controversially, these treatments have been excluded from classification as regenerative endodontic techniques (Murray *et al.*, 2007), which appears at odds with the recent American Association of Endodontics (AAE) definition of regenerative endodontics, 'biologically-based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp dentin complex' (AAE, 2012). Clearly this definition places pulp capping firmly in this category.

#### **1.1.4 Challenges associated with current pulp preservation treatments**

Traditional pulp capping materials, such as calcium hydroxide [Ca(OH)<sub>2</sub>] and more recent resin-based composites (RBCs), have limited success in part due to their inability to prevent microleakage at the tooth-material interface (Bergenholtz *et al.*, 1982; Hilton *et al.*, 2002) as well as the toxicity of their constituents (Mantellini *et al.*, 2003; Pananjpe *et al.*, 2008). The development of materials, such as mineral trioxide aggregate (MTA) (Fig. 1.2) and Biodentine, have offered improvements over existing materials, demonstrating superior histological responses (Nair *et al.*, 2008; Nowicka *et al.*, 2015), as well as improved clinical outcomes (Mente *et al.*, 2014). Reportedly, Ca(OH)<sub>2</sub> stimulates a reparative response by non-specific mechanisms (Sangwan *et al.*, 2013), which appear to involve a modulation of pulpal inflammation and the creation of an environment conducive to tissue repair. The non-specific nature of the response to these materials creates problems for scientists and clinicians in elucidating both the specific response to material application and also, in devising targeted repair solutions. Ca(OH)<sub>2</sub> exhibits other limitations as the hard tissue bridges beneath these



**Figure 1.2 Histological response to pulp capping.** **i.** Macrophotographic view of the mesial half of a human maxillary third molar demonstrating the remnants of a restorative material (A) and MTA™ capping material (B) at 1 month. Note the distinct hard tissue bridge (arrow) (original magnification X8). **ii.** Photomicrograph of a histological section of the specimen in **i.** of a MTA™ pulp cap at 1 month. Note that the mineralised barrier (arrow) stretches across the entire width of the exposed pulp (C) (original magnification ×16). **iii.** Higher magnification photomicrograph from **i & ii.** Cuboidal cells (arrows) line the hard tissue barrier (D), note the absence of inflammatory cells in the pulp (E) (original magnification ×85). **iv.** Photomicrograph of a selected serial section of hard-setting cement (Dycal™) at 1 month. Engorged blood vessels are prominent and inflammatory cells are present. Note the presence of Dycal™ particles (arrows) in the pulp (F) (original magnification ×16). Images adapted from Nair *et al.*, 2008.

dressings are generally incomplete and contain 'tunnel' defects (Fig. 1.2) that are often associated with inflammation or necrosis (Cox *et al.*, 1996). Although MTA induces more complete hard tissue barriers (Nair *et al.*, 2008), limitations with regard to handling concerns and post-operative tooth discolouration have been reported (Felman & Parashos, 2013).

#### **1.1.5 Opportunities for new therapies**

Currently available dental restorative materials do not fulfil the 'ideal' properties of a pulp capping material (Scarano *et al.*, 2003), namely to; i) maintain the vitality of the dental pulp, ii) promote the formation of a mineralised bridge, iii) possess appropriate mechanical properties, iv) form an adhesive bond to dentine (prevent microleakage), and v) be insoluble and be easy to handle clinically. The limitations of current pulp capping materials have driven the development of targeted bio-inductive alternatives that can both prevent microleakage and promote dentine-pulp regenerative events (Ferracane *et al.*, 2010). Augmenting existing dental restorative materials with anti-oxidants (Kojima *et al.*, 2008; Paranjpe *et al.*, 2008), antibiotics (Imazato *et al.*, 2007; Kamocki *et al.*, 2015), the application of growth factors (Smith, 2003; Zhang *et al.*, 2011) and gene therapy approaches (Nakashima *et al.*, 2003) have all been proposed as potential approaches to generate biologically-focussed dental materials. While growth factor and gene therapy solutions hold considerable future promise, they are limited by expense, dose-response effects, carrier issues and practical applicability (Tzafias, 2004; Smith *et al.*, 2008). The potential use of relatively inexpensive readily available epigenetic-modifying agents to influence SC function and promote mineralisation has been recently highlighted in other disciplines (Schroeder & Westendorf, 2005; Humphrey *et al.*, 2008) and may provide a bio-inductive material

solutions in dentistry (Duncan *et al.*, 2011). Currently, however, their pulpal effects have not been thoroughly elucidated within restorative dentistry.

## **1.2 Epigenetics**

Deoxyribonucleic acid (DNA) is condensed within the nucleus of all eukaryotic cells in a repeating nucleosome structure, which represents 147 base pairs of DNA tightly bound to a group of histone proteins to form chromatin (Luger, 2003; Hake *et al.*, 2004). Each nucleosome contains two copies of each of the four core histone proteins (H2A, H2B, H3 and H4), while the nucleosomes are bound together by a range of proteins including the linker histone H1 (Kornberg & Lorch, 1999). A feature of the histone proteins is the presence of positively charged 'tails', high in arginine or lysine residues, which are frequently subject to post-translational modifications such as acetylation, methylation and phosphorylation (Spange *et al.*, 2009; Gordon *et al.*, 2015). Although every human cell contains the same genetic information, not all genes are expressed at any given time (Portela & Esteller, 2010) with transcription tightly orchestrated by complex molecular mechanisms, which enable certain genes to be expressed and others suppressed depending on cellular and tissue requirements (Horn & Peterson, 2002). Molecular signalling alters gene transcription by modifying the conformation of the chromatin structure, which dynamically condenses and relaxes limiting or permitting transcription factor access to the DNA (Kleff *et al.*, 1995; Margueron *et al.*, 2005; Vaissière *et al.*, 2008). This chromatin remodelling can be achieved by several mechanisms, including covalent alteration of core histone 'tails', modification of the DNA itself and disruption of the nucleosome structure (Hake *et al.*, 2004).

These epigenetic 'tags' (or modifications) of chromatin are generally defined as alterations, which do not change the DNA sequence, but affect the chromatin architecture and its accessibility resulting in an alteration to gene expression that is heritable through cell division (Barros & Offenbacher, 2009; Arnsdorf *et al.*, 2010; Cardoso *et al.*, 2010). This epigenetic concept, while currently topical, actually dates back to 1942 when Conrad Waddington introduced the term 'epigenetic landscape' to describe the molecular mechanisms responsible for converting genetic information into a specific phenotype (Waddington, 1942). Our scientific understanding of the cellular role of epigenetics has evolved into a detailed analysis of the effect of a range of modifications on chromatin architecture, the effects on gene (Jaenisch & Bird, 2003) and non-coding ribonucleic acid (RNA) expression (Gloss & Dinger, 2016) and subsequent influences on cellular phenotype (Bostock *et al.*, 2009). As a result, epigenetic modifications of chromatin structure have been the focus of considerable research interest recently due to their roles in disease aetiology and their potential ability to be targeted and reversed by therapeutic strategies (Kelly *et al.*, 2010; Portela & Esteller, 2010; Marks, 2010).

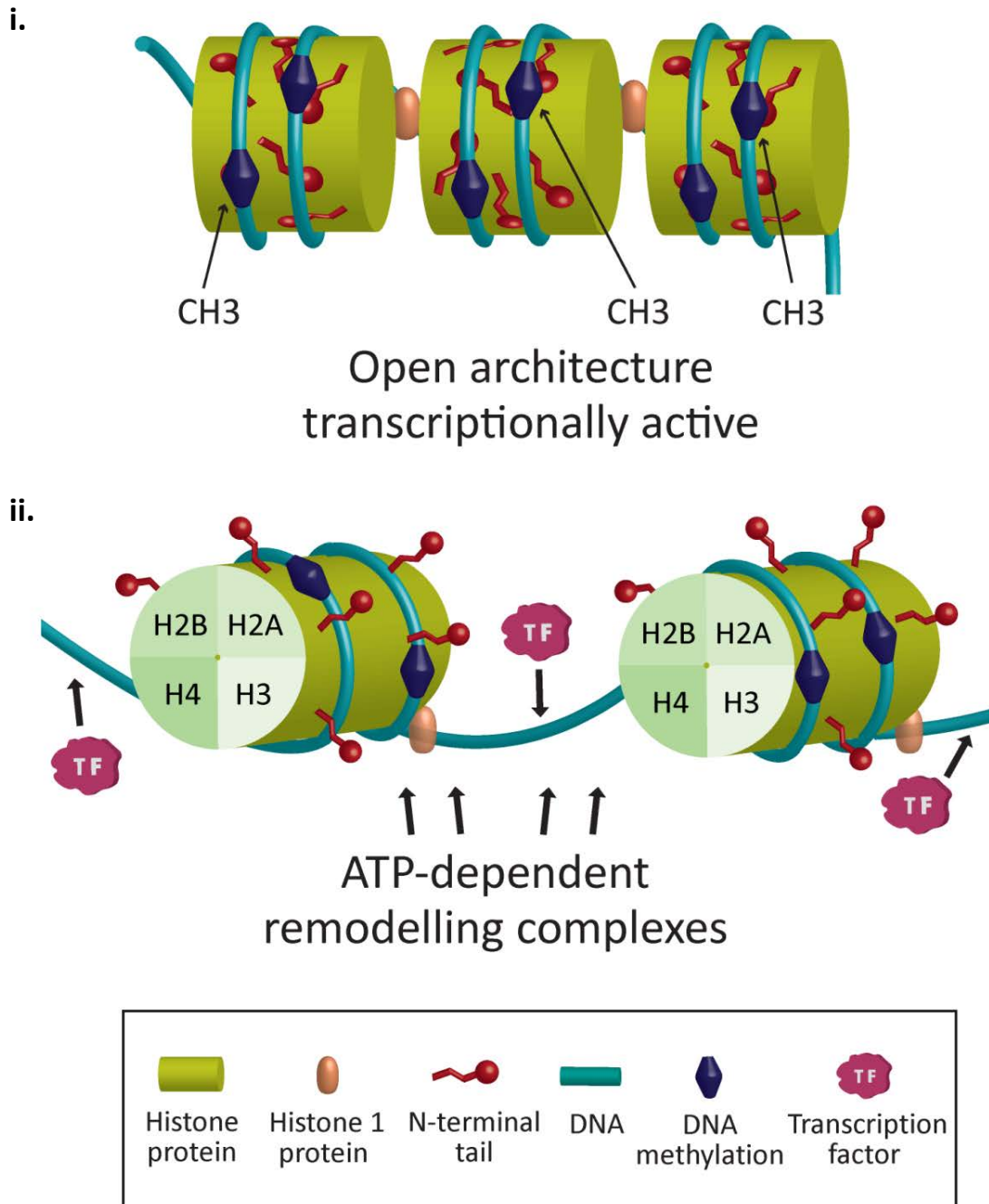
### **1.2.1 Epigenetic modifications**

The two principal types of epigenetic modifications are; i) DNA methylation (Nagase & Ghosh, 2008) and ii) post-translational modification of histone proteins (Vaissière *et al.*, 2008) (Fig. 1.3). Other less characterised epigenetic modifications have also been described including nucleosome positioning (Portela & Esteller, 2010) and chromatin's organization of functional regulatory domains (Lian, 2015). Although it is useful from a didactic perspective to categorise epigenetic modifications and study their influence in isolation, it is likely that

physiologically a complex interplay will exist between modifications, such as histone acetylation and phosphorylation and DNA methylation (Daujat *et al.*, 2005; Milutinovic *et al.*, 2007; Ou *et al.*, 2007; Vaissière & Herceg, 2010).

#### **1.2.1.1 DNA methylation**

The most studied epigenetic modification is the methylation of CpG paired residues directly on DNA (Portela & Esteller, 2010), which results in the covalent attachment of a methyl group (-CH<sub>3</sub>) to the carbon-5' position of the cytosine ring (Illingworth & Bird, 2009) (Fig. 1.3). Until recently, it was understood that methylation exclusively occurred in specific regions of DNA where CpG dinucleotides were clustered, termed CpG islands. However, methylation events have been demonstrated to occur more widely at other sites in embryonic SCs (ESCs) (Lister *et al.*, 2009). Notably, CpG islands are not evenly distributed throughout the genome, but tend to locate in gene promoter regions (Cooper *et al.*, 1983; Larsen *et al.*, 1992), which support the hypothesis that heritable DNA methylation, could provide a mechanism for the developmental regulation of gene expression (Riggs, 1975; Holliday & Pugh, 1975). Indeed, CpG island-methylation tends to be characterised by a transcriptionally repressive state and gene silencing (Weber *et al.*, 2007). Notwithstanding, it is an oversimplification to suggest that hypermethylation has a direct correlation with the suppression of associated genes as this relationship remains relatively weak (Oakes *et al.*, 2007).



**Figure 1.3 Diagram of principal epigenetic modifications.** i. Methylation ( $-CH_3$ ) of chromosomal DNA, wrapped around the histone protein octamer, generally occurs on cytosine residues at specific regions called CpG islands, which generally overlap gene promoter regions. DNA methylation changes are considered stable epigenetic modifications and are catalysed by DNA methyltransferase enzymes. The methylation status of DNA is considered key to regulating cellular transcription processes. ii. Histone modifications, including acetylation, of positively charged acetyl tails alters the conformation of chromatin promoting gene expression and pleiotropic cellular effects. In contrast to methylation changes these are considered labile modifications, thereby presenting an attractive therapeutic target. The epigenetic modification of these core proteins is enzymatically controlled by histone deacetylase (HDAC), histone acetyl transferase (HAT) and histone demethylase enzymes. The nucleosome structure can move along the linker DNA strand facilitating changes in gene transcriptional activity.



Individual DNA methylation patterns are established and maintained by the cellular enzymes, DNA methyltransferases (DNMTs), which transfer a -CH<sub>3</sub> group to the cytosine residue from the universal methyl donor S-adenosyl-L-methionine (Jin & Robertson, 2013). There are five members of the DNMT family (DNMT-1, -2 -3a, -3b, -3l), which establish stable epigenetic DNA 'tags' and from a therapeutic perspective, are potentially reversible (Holliday & Pugh, 1975). Following DNA methylation, the exposed -CH<sub>3</sub> groups on the cytosine nucleotides inhibit transcription directly by precluding the recruitment of DNA binding proteins (Kuroda *et al.*, 2009). Subsequently, methyl-CpG-binding domain proteins are attracted to the DNA and act in synchrony with histone deacetylase (HDAC) enzymes and remodelling complexes to alter chromatin architecture (Portela & Esteller, 2010). DNA methylation processes are considered essential for mammalian development (Robertson, 2005) with an increase in DNA-methylation broadly leading to an inhibition of gene expression (Auclair & Weber, 2012), while demethylation promotes transcriptional activity (Narlikar *et al.*, 2002). It appears that DNMTs are essential for genomic integrity and disruption of their activity has been linked to chromosome instability, neurodegenerative diseases and neoplasia (Goelz *et al.*, 1985; Urduingio *et al.*, 2009).

Methylation status is altered in cancerous cells and is characterised by severe global hypomethylation, although hypermethylation at certain CpG islands has also been reported (Goelz *et al.*, 1985). From a therapeutic endodontic perspective, a link between methylation status and inflammation is emerging in medicine and dentistry (Bayarsaihan, 2011) and has recently been a focus of attention in the dental pulp (Cardoso *et al.*, 2014) and periapical area of the tooth (Campos *et al.*, 2016). A beneficial effect of DNMT inhibitors and the

promotion of mineralisation processes have recently also been demonstrated in both bone (Yan *et al.*, 2014) and dental pulp studies (Zhang *et al.*, 2015).

#### **1.2.1.2 Histone modifications**

Core histones can be modified by methylation, acetylation, phosphorylation, ubiquitination and SUMOylation (Zhang & Reinberg, 2001; Kelly *et al.*, 2010). However, in contrast to the stability of DNA methylation changes, histone modifications are considered more labile epigenetic 'tags', being readily reversible (Kelly *et al.*, 2010). Histone tail modification induces pleiotropic cellular effects including transcription, DNA repair processes and chromosomal condensation, with notably the facilitation or inhibition of transcription being dependent on both the histone residue affected and the specific modification type (Kouzarides, 2007; Huertas *et al.*, 2009). These covalent modifications are generally controlled homeostatically by enzymatic activity, with an imbalance leading to aberrant histone alteration, which is evident in disease states (Jones & Baylin, 2007). The enzymatic balance and the reversible nature of the histone modifications, however, also provide an opportunity for therapeutic intervention (Bolden *et al.*, 2006). As a result, histone deacetylation (Grant *et al.*, 2007) and more recently histone methylation inhibitors, have been investigated in clinical trials for the treatment of cancer, while preclinical trials using deacetylation inhibitors are ongoing for the treatment of inflammatory and neurodegenerative disorders (Maes *et al.*, 2015; Momparler & Côté, 2015).

#### **1.2.1.2.1 Methylation**

Histone proteins are methylated on arginine and lysine residues only (Zhang & Reinberg, 2001). In addition, the transcription effects of methylation are also residue-dependent with trimethylation of certain histone residues, H3K4 and H3K79, being associated with a transcriptionally active chromatin state, while high methylation levels of other residues, H3K27 and H4K79, are associated with inactivity (Li *et al.*, 2007). Altered methylation patterns have been established in disease states with demethylation of H4K20me<sub>3</sub> associated with cancer (Jones & Baylin, 2007), while increased methylation of H3K9me<sub>3</sub> was linked to Huntington's disease (Urduingio *et al.*, 2009). Different epigenetic modifications appear to be interrelated, with high levels of acetylation linked to the high levels of H3K4 and H3K36 methylation and an open euchromatin state (Li *et al.*, 2007). There is also an established interrelationship between histone lysine methylation and DNA methylation processes during developmental processes (Rose & Klose, 2014). The enzymes which catalyse the modifications, histone methyltransferases and histone demethylases, are specific to individual histone subunits and residues (Kouzarides, 2007), but can also methylate non-histone proteins (Huang & Berger, 2008). Interestingly, as histones can be methylated at several sites simultaneously it appears that it is the combination of marks that determine the overall influence on transcription and cell phenotype (Wang *et al.*, 2008).

#### **1.2.1.2.2 Acetylation**

Acetylation of lysine residues in the positively charged histone 'tail' is the most clearly elucidated histone modification (Philips, 1963) and is associated with an open transcriptionally active chromatin state; conversely, deacetylation is associated with gene

silencing (Kleff *et al.*, 1995; Taunton *et al.*, 1996). The conformational relaxation of the tightly bound chromatin structure creates a remodelled structure in which proteins can access DNA to facilitate transcription and gene expression (Grewal & Moazed, 2003; Hake *et al.*, 2004). Two groups of balancing enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs), control the homeostatic balance and alterations in this balance affect gene expression (Yang & Seto, 2008). HDACs are also active on a plethora of non-histone substrates, including transcription factors and signalling mediators (Glozak *et al.*, 2005; Lee & Workman, 2007) and as a result, the term deacetylases may be a better descriptor for their cellular function (Balasubramanian *et al.*, 2009).

#### **1.2.1.2.3 Other histone modifications**

Although phosphorylation, ubiquitination and SUMOylation of the histone tails of core proteins are less well elucidated than histone methylation and acetylation, these modifications play crucial roles in DNA repair, replication and the regulation of chromosome conformation (Kourzarides *et al.*, 2007). Indeed, the role of ubiquitination in pathology (Maragoui *et al.*, 2015), as well as SUMOylation in cancer and neurodegenerative diseases has recently been highlighted (Eifler & Vertegaal, 2015). To date it remains unclear as to why there is a relative lack of information regarding these modifications. However, authors have postulated many theories including the relative infrequency with which these modifications occur (Portela & Esteller, 2010), their poorly characterised role in disease (Montecino *et al.*, 2015) and the absence of therapeutic inhibitors (Kelly *et al.*, 2010). It appears inevitable that future research will demonstrate cross-talk and interplay between methylation, acetylation and other histone modifications as previously illustrated by the

balancing cellular effects of histone methylation and phosphorylation in controlling gene expression (Daujat *et al.*, 2005).

### **1.2.1.3 Other epigenetic modifications**

In addition to DNA methylation and a range of histone modifications the position of the nucleosome on the cellular DNA is critical for regulating both gene and noncoding RNA expression.

#### **1.2.1.3.1 Nucleosome positioning**

The nucleosome structure provides a physical barrier to transcription factors and associated co-mediator's gaining direct access to the DNA, while the nucleosome's position within certain regulatory genomic regions also creates an environment to permit or prevent transcription (Portela & Estrella, 2010). To enable dynamic access to the DNA, large macromolecular families called chromatin remodelling complexes, move, destabilise and restructure nucleosomes in a manner dependent on ATP hydrolysis, exerting a repressive or permissive transcriptive affect often in combination with HDACs (Clapier & Cairns, 2009). Epigenetic interactions between DNA methylation (Harikrishnan *et al.*, 2005) and histone modifications (Wysocka *et al.*, 2006) at specific genomic loci appear to destabilise the nucleosomes location, regulating the complex remodelling activity, which subsequently alters transcription factor access and associated gene expression (Bourachot *et al.*, 2003). Although for the purposes of illustrating the range of epigenetic modifications, nucleosome positioning is considered a separate category, it is apparent that the dynamics of

nucleosome position are orchestrated by a network of other epigenetic influences (Zentner & Henikoff, 2013).

#### **1.2.1.3.2 Non-coding RNA**

Non-coding RNA (ncRNA) molecules which are transcribed from DNA, but are not translated into a protein, are considered key regulators of epigenetically controlled gene expression (Kelly *et al.*, 2010; Lian, 2015). Within the ncRNA family, selected types are involved in facilitating the epigenetic control of gene expression and are sub-classified as short-ncRNAs (sncRNAs), which include microRNA (miRNA), small interfering RNA (siRNA), and long-ncRNAs (lncRNAs) (Mercer & Mattick, 2013). Over the last 10 years, the knowledge of these ncRNAs, in particular miRNA, has developed in relation to; i) epigenetic control due to their dysregulation in diseases such as cancer (Chawla *et al.*, 2015), ii) regulation of epigenetic mechanisms, such as DNA methylation (Fabbri *et al.*, 2007) and iii) expression, which conversely, is regulated by epigenetic modifications (Saito *et al.*, 2006). Additionally, lncRNAs have also been shown to epigenetically modulate chromatin architecture by recruiting and binding chromatin-modifying proteins to specific genomic sites (Khalil *et al.*, 2009), while also guiding proteins, such as histone methyltransferases, to those sites (Nagano *et al.*, 2008; Guttman *et al.*, 2011). Notably, recent studies have begun to demonstrate the importance of miRNA expression and gene expression regulation within pulpal mineralisation responses (Heair *et al.*, 2015; Sun *et al.*, 2015).

#### 1.2.1.4 Stem cell epigenetics

Classically, ESCs exhibit an open chromatin structure characterised by relatively low levels of DNA methylation and high levels of histone acetylation (Mohammad & Baylin, 2010). Following specialised cell lineage commitment and differentiation, the chromatin is modified to a more tightly bound conformation with reducing levels of acetylation and increasing levels of DNA methylation (Surani *et al.*, 2007). It is now evident that ESCs are maintained in a self-renewing state by a complex network of transcription factors (Jaenisch & Young, 2008), while differentiation and developmental potential are under epigenetic control (Meissner, 2010). During differentiation, the pluripotency genes Oct4 and Nanog, are repressed (Yamanaka & Blau, 2010), an effect which is mediated initially by histone modifications, with the downregulation maintained and cell reprogramming prevented, by DNA methylation events (Feldman *et al.*, 2006).

While it was considered that DNA cytosine methylation occurred exclusively at CpG islands, recent studies noted that up to one-quarter of all ESC methylation was identified in a non-CG context (Lister *et al.*, 2009). Indeed, the observed pattern of non-CG methylation disappeared when differentiation processes were induced in ESCs supporting a crucial role for methylation in determining cell fate (Lister *et al.*, 2009). Further characterisation of epigenetic modifications in ESCs has identified hypermethylation at 3% of promoter regions in CpG islands, notably in developmental genes such as *Rhox2* (Fouse *et al.*, 2008). It has been proposed that although global methylation levels of CpG may be similar between cell types, the distributions of methylation marks in ESCs are unlike any other cell type (Meissner *et al.*, 2008). Other epigenetic modifications are also important regulators of SC function and self-renewal with HDAC activity and acetylation status vital to the self-renewal capabilities of

mesenchymal SCs (Romagnani *et al.*, 2007; Lee *et al.* 2009) by maintaining the expression of key pluripotent transcription factors (Jamaladdin *et al.*, 2014). Acetylation and methylation marks in SCs are controlled by cellular mediators, including the polycomb and trithorax protein complexes, which occupy ESC gene promoter sites and catalyse specific histone modifications (Guenther & Young, 2010; Brien *et al.*, 2012).

Recently, interest in the epigenetic control of DPSC behaviour has led to the suggestion that dental developmental anomalies, such as dentine dysplasia and dentinogenesis imperfecta, may be related to epigenetic modifications present during odontoblast differentiation (Sun *et al.*, 2015). However, current research approaches are preliminary and have not, to date, analysed pure or enriched SC populations (Cardoso *et al.*, 2014). In a recent SC study, epigenetic states and related differentiation profiles were analysed in SC populations of DPSCs and dental follicle progenitor cells (DFPCs), by their expression of odontogenic genes, including dentine sialophosphoprotein (DSPP) and dentine matrix protein-1 (DMP-1). Transcript levels were epigenetically-suppressed in DFPCs, while osteogenic stimulation *in vitro* demonstrated significant mineralisation increases only in DPSCs (Gopinathan *et al.*, 2013). Interestingly, a highly dynamic histone modification response was demonstrated in mineralising DFPCs, but not in DPSCs, with DPSCs also expressing relatively high levels of the pluripotency-associated transcripts, Oct4 and Nanog. It was concluded that these two neural crest-derived SC populations were distinguished by epigenetic repression of dentinogenic genes with dynamic histone enrichment in DFPCs during mineralisation. The study highlighted the potential important role of epigenetic control within the pulp's terminal differentiation processes.



### **1.2.1.5 Epigenetic modifications as therapeutic targets in the dental pulp**

For the biologically-based endodontic procedures of direct pulp capping and partial pulpotomy, to be successful, an environment conducive to tissue repair must be established. Furthermore, inflammatory control as well as the promotion of mineralisation, angiogenesis and neurogenesis are all necessary to enable effective repair and healing (Grando Mattuella *et al.*, 2007; Cooper *et al.*, 2010). Potentially, epigenetic-modifying agents targeting DNA methylation and histone acetylation could play a role in regenerative endodontics as they have previously been shown to be effective in reducing inflammation, promoting mineralisation and modulating regenerative processes in a range of cell types (Shanmugam & Sethi, 2013; Gordon *et al.*, 2015; Zhang *et al.*, 2015). Indeed, epigenetic modifications present attractive therapeutic targets due to their association with disease and also because they are relatively easy to reverse pharmacologically (Kelly *et al.*, 2010; Gordon *et al.*, 2015). Within dentistry, it has been postulated that the epigenetic reprogramming which accompanies the viral reprogramming of somatic cells to induced pluripotent SCs (iPSCs) (Takahashi & Yamanaka, 2006; Huangfu *et al.*, 2008), may prove an important tool for wound-healing or regeneration in periodontal tissues (Barros & Offenbacher, 2009 & 2014). For the reasons described above, epigenetic modification is an area of significant interest within periodontology (Grover *et al.*, 2014) and endodontics (Duncan *et al.*, 2011)

The enzymes that regulate epigenetic chromatin modifications including methyltransferases, demethylases, HATs and HDACs, are of particular therapeutic interest. HDACs have been demonstrated to be a particularly attractive target as they have been associated with the regulation of mineralisation and developmental cellular processes (Gordon *et al.* 2015), while also being readily inhibited pharmacologically (Richon *et al.*,

1996). Eighteen human HDACs have been identified, which have been categorised into four classes, with classes 1, 2, and 3 containing zinc dependent enzymes (Gregoretto *et al.*, 2004). HDAC classes exhibit different cellular locations (Montgomery *et al.*, 2007), tissue expressions (Klinz *et al.* 2012) and are associated with different cellular processes (de Ruijter *et al.*, 2003, Witt *et al.*, 2009, Jamaladdin *et al.*, 2014). Histone deacetylase inhibitors (HDACis) also represent exciting therapeutic candidates as their alterations of gene expression patterns modulate intracellular signalling, with subsequent effects on cell phenotype. The medical literature also reports that HDACis are associated with anti-inflammatory effects, pro-mineralisation, increased SC differentiation and improved regenerative responses (Halili *et al.*, 2009; Xu *et al.*, 2009; Wang *et al.*, 2010). Consequently, HDACis have the potential to enhance tertiary dentinogenesis by influencing the cellular and tissue processes critical to the success of vital pulp treatment. Furthermore, HDACi-induced modifications occur at nano to micro molar concentrations with minimal side effects and therefore, may offer the ability to develop an easily placed, inexpensive bio-inductive dental restorative material.

### **1.3 Hypotheses**

The hypotheses to be examined in the current investigations are that:

- HDACis can positively affect cell proliferation and differentiation to accelerate mineralisation events in dental pulp-derived cells.
- HDACis induce reparative responses in primary DPCs at concentration and dose levels which do not stimulate significant anti-proliferative, apoptotic or necrotic effects.

- The mechanisms behind the regenerative properties of a specific HDACi, namely suberoylanilide hydroxamic acid (SAHA), relate to transcriptomic changes which modulate selected mineralisation-associated pathways and the novel markers identified interact with SAHA to promote and augment pulpal reparative processes.
- HDACis remove or alter the smear layer remaining after dentine preparation owing to their acidic nature.
- HDACi solubilise a range of dentine matrix components (DMCs), which may promote bioactive and clinically relevant dental repair responses.

#### **1.4 Aims and objectives**

The overall aim of this body of work was to assess the ability of one group of epigenetic-modifying agents, namely HDACis, to induce mineralisation and promote reparative responses in DPCs. The specific objectives to be examined include to:

- Review the literature in relation to HDACi cellular function, focusing on HDACi-induced effects including inflammation control, mineralisation-induction, and wound repair, which may be beneficial for therapeutic application within the pulp.
- Summarise the current knowledge of all epigenetic-modifying agents, in particular HDACis, to consider their role in SC differentiation, de-differentiation and reprogramming in the context of the development of novel regenerative therapies for the dentine-pulp complex.
- Investigate the effects of two pan-HDACis, namely trichostatin A (TSA) and valproic acid (VPA), on cell viability, cell cycle and mineralisation responses in dental-papillae derived cell-line cultures.

- Analyse HDACi-induced mineralisation responses in primary DPC cultures, focusing on cell viability, growth, apoptosis mineralisation-associated gene/protein marker expression.
- Identify a response range of HDACi concentrations and doses in primary DPCs in line with previous studies (Ungerstedt *et al.*, 2005; Lee *et al.*, 2010) where significant differences between transformed and primary cell culture responses to HDACis were reported.
- Determine the mechanisms by which the clinically-approved HDACi, SAHA, promotes regenerative processes in primary DPCs using high-throughput transcriptomic analyses, prior to pathway analysis, molecular validation and pharmacological inhibition approaches.
- Review the literature on the roles of bioactive molecules located in the dentine-pulp complex, their potential contribution to pulpal reparative events after injury and as a part of regenerative therapeutic strategies.
- Compare the solubilisation of a range of DMCs using three HDACis with characterised extractants, to identify conditions which may favour bioactive dentine-pulp repair responses if HDACis were to be applied clinically in regenerative endodontic treatment protocols.

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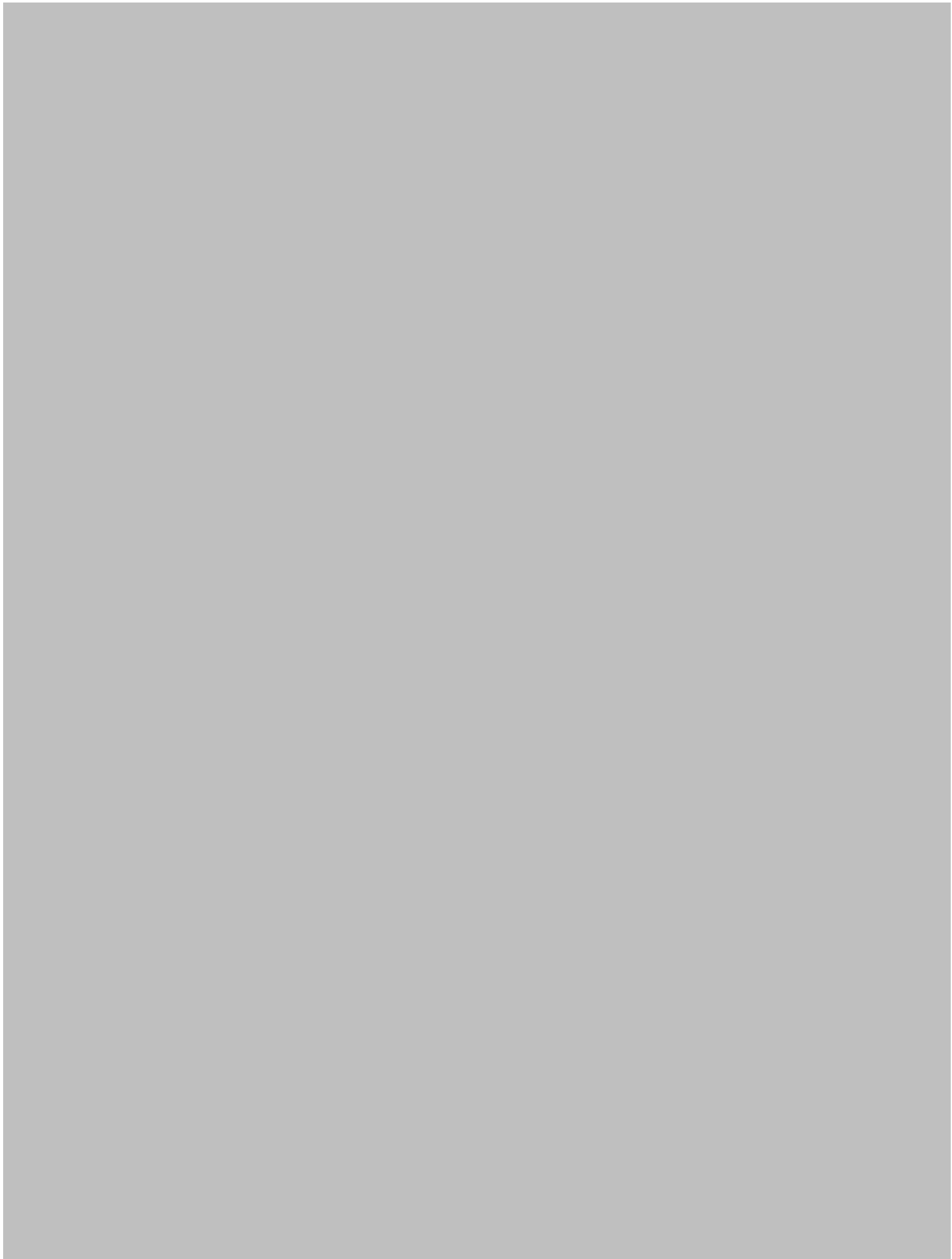
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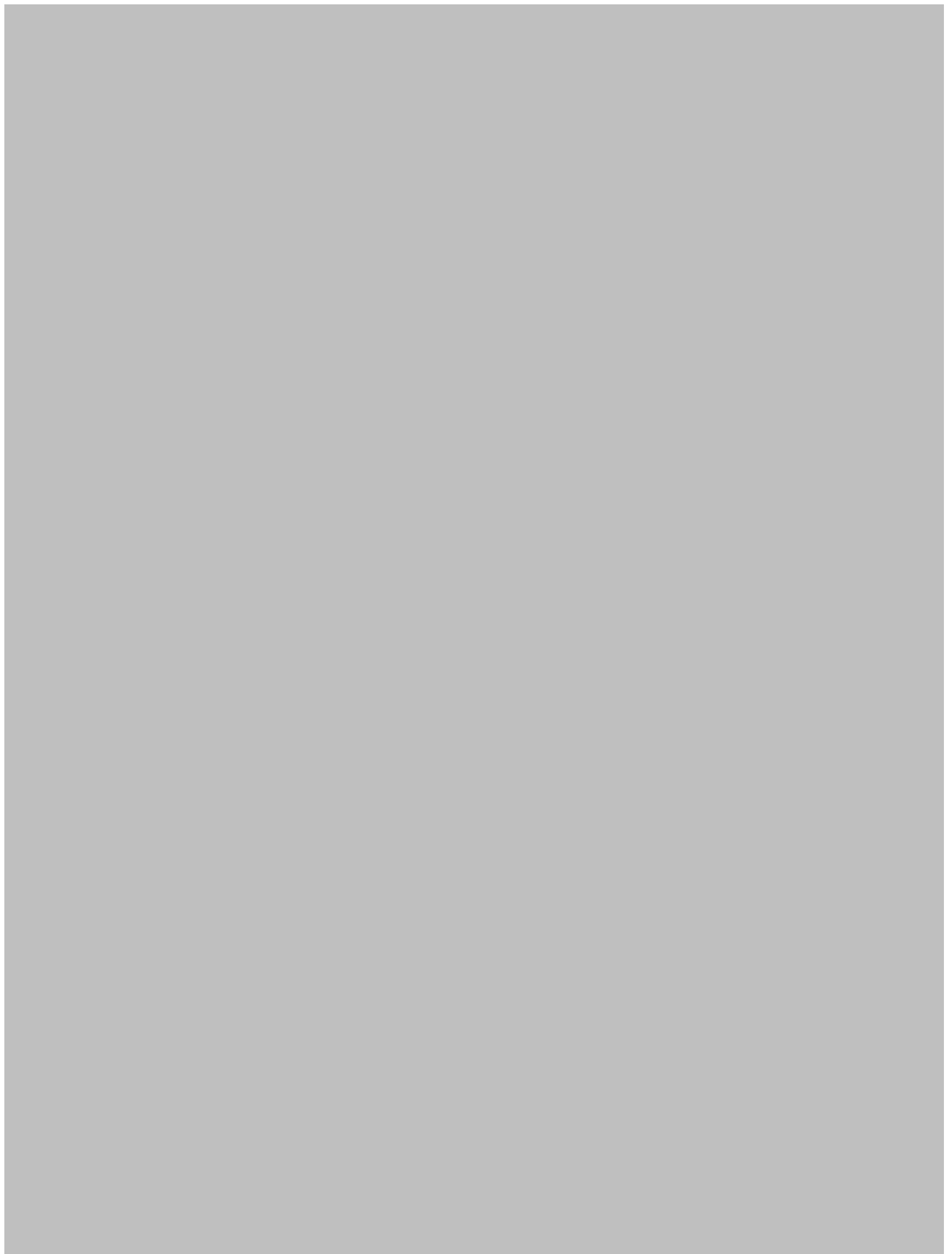
## CHAPTER 2

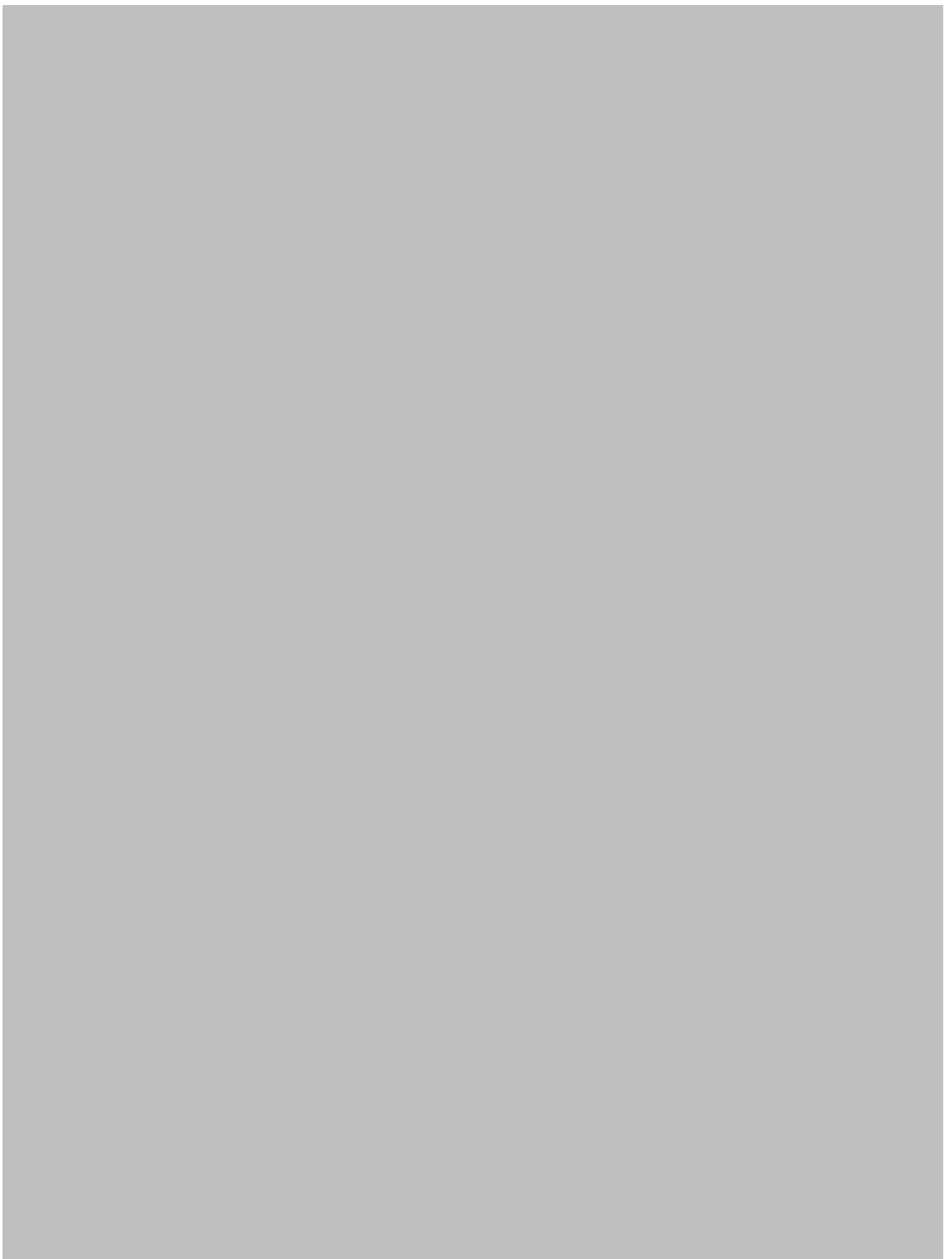
### PUBLICATION 1: REVIEW

**Duncan HF, Smith AJ, Fleming GJP, Cooper PR. (2011). HDACi: cellular effects, opportunities for restorative dentistry. *J Dent Res* 90:1377-1388.**

In the context of the planned body of work, the medical and cell biology literature was reviewed in relation to the influence of epigenetic-modifications, histone acetylation, HDAC enzymes and the use of one specific group of epigenetic modifying agents, HDACis, in altering cell phenotype. The bulk of the review focussed on the role of HDACis as a modulator of pleiotropic cellular processes, such as differentiation, proliferation, apoptosis, reactive oxygen species generation, neurogenesis and angiogenesis. Thereafter, the potential translational benefits of the HDACi-induced effects specific to pulp tissue injury and repair were reviewed to include inflammatory control, mineralisation and tissue engineering, pain and stem cell/regenerative effects. HDACi associated side-effects, barriers to clinical implementation and the need for future research were also considered.



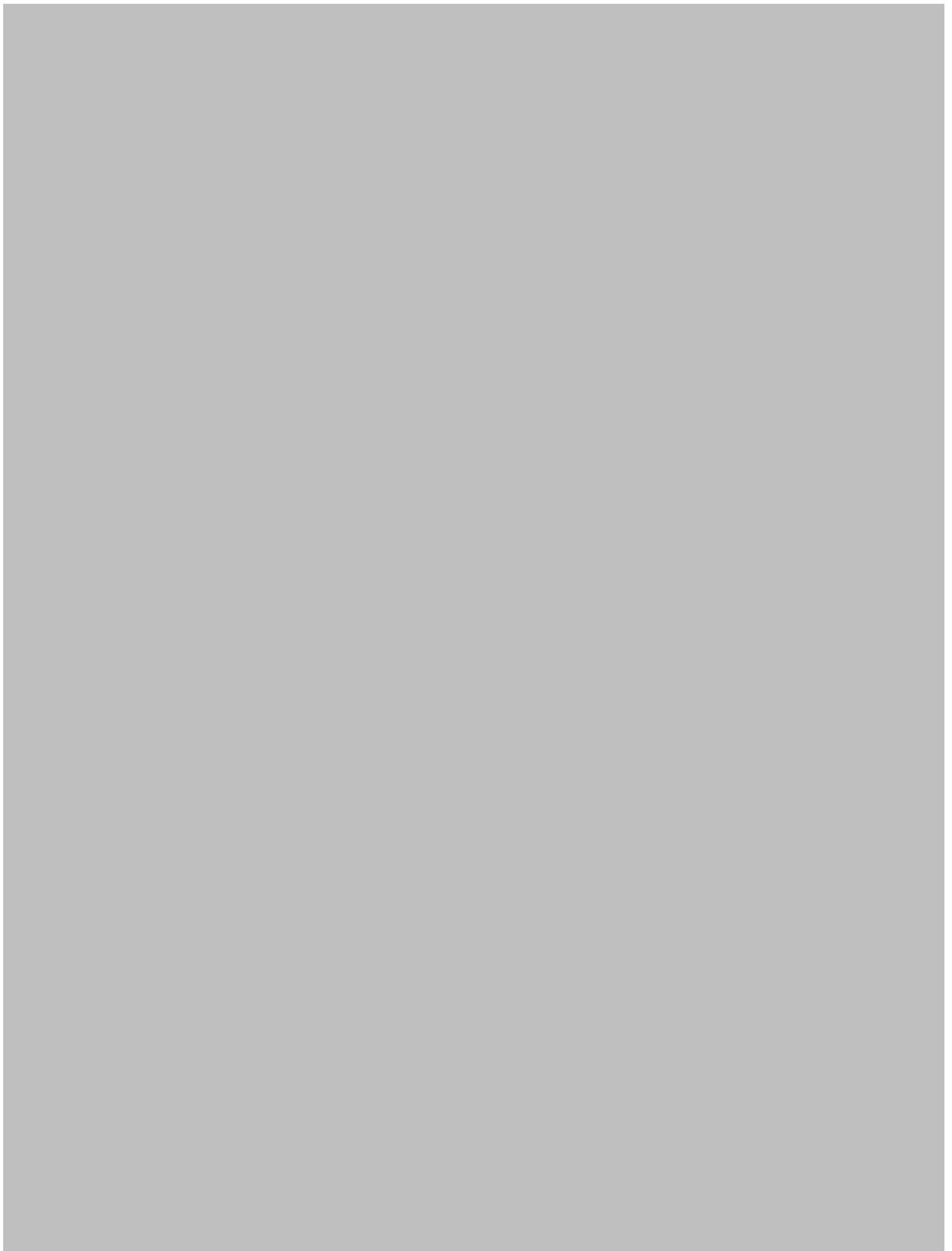


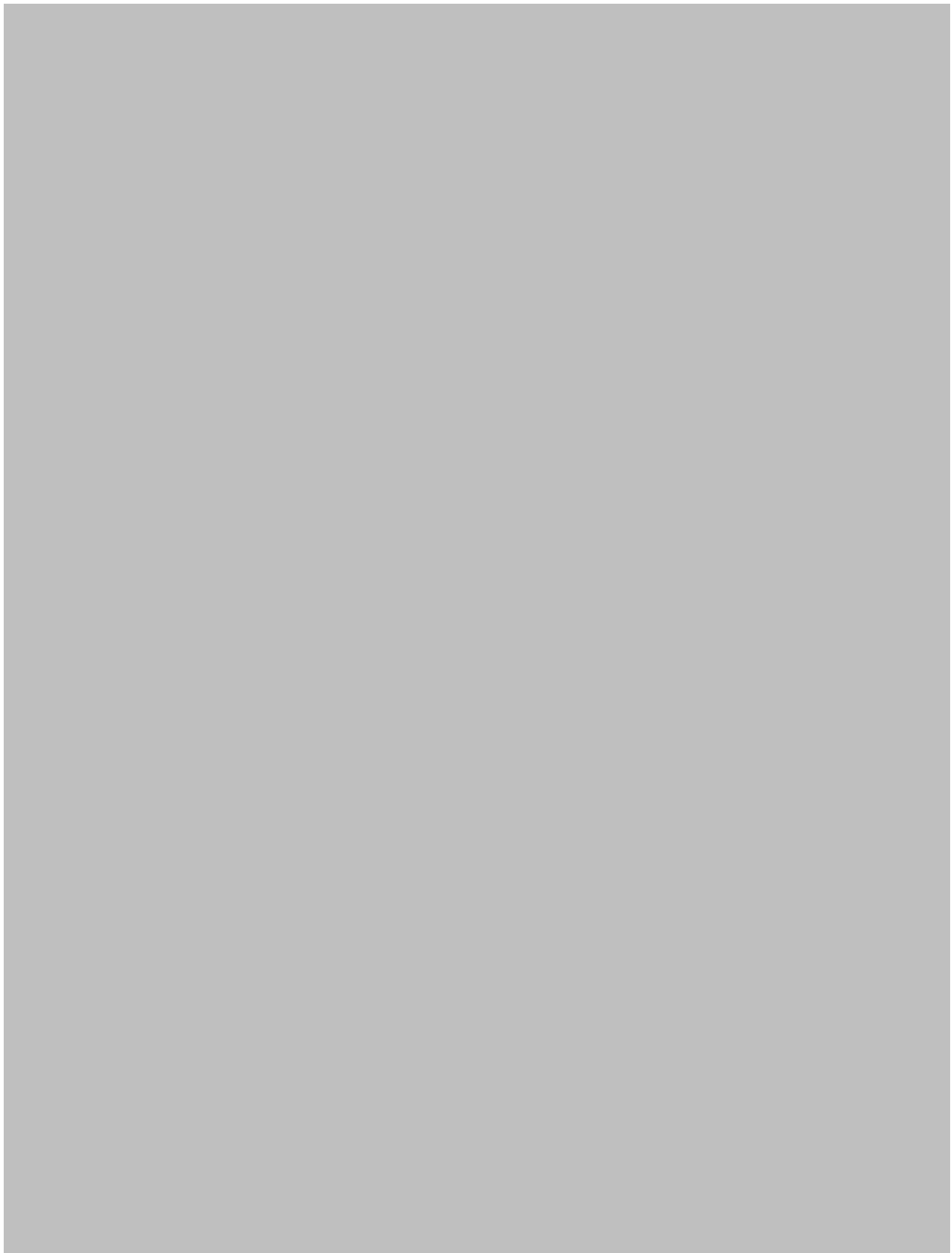












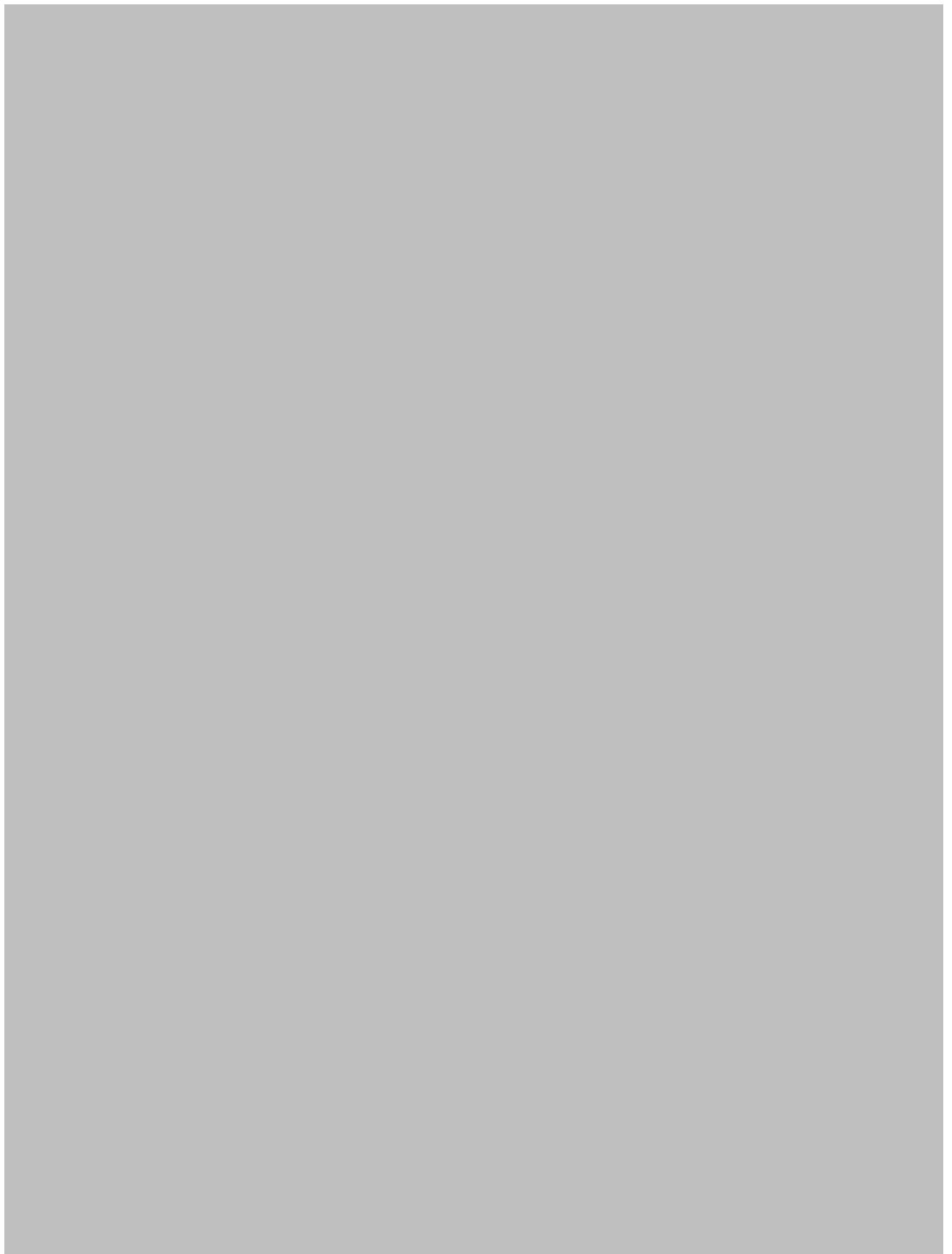


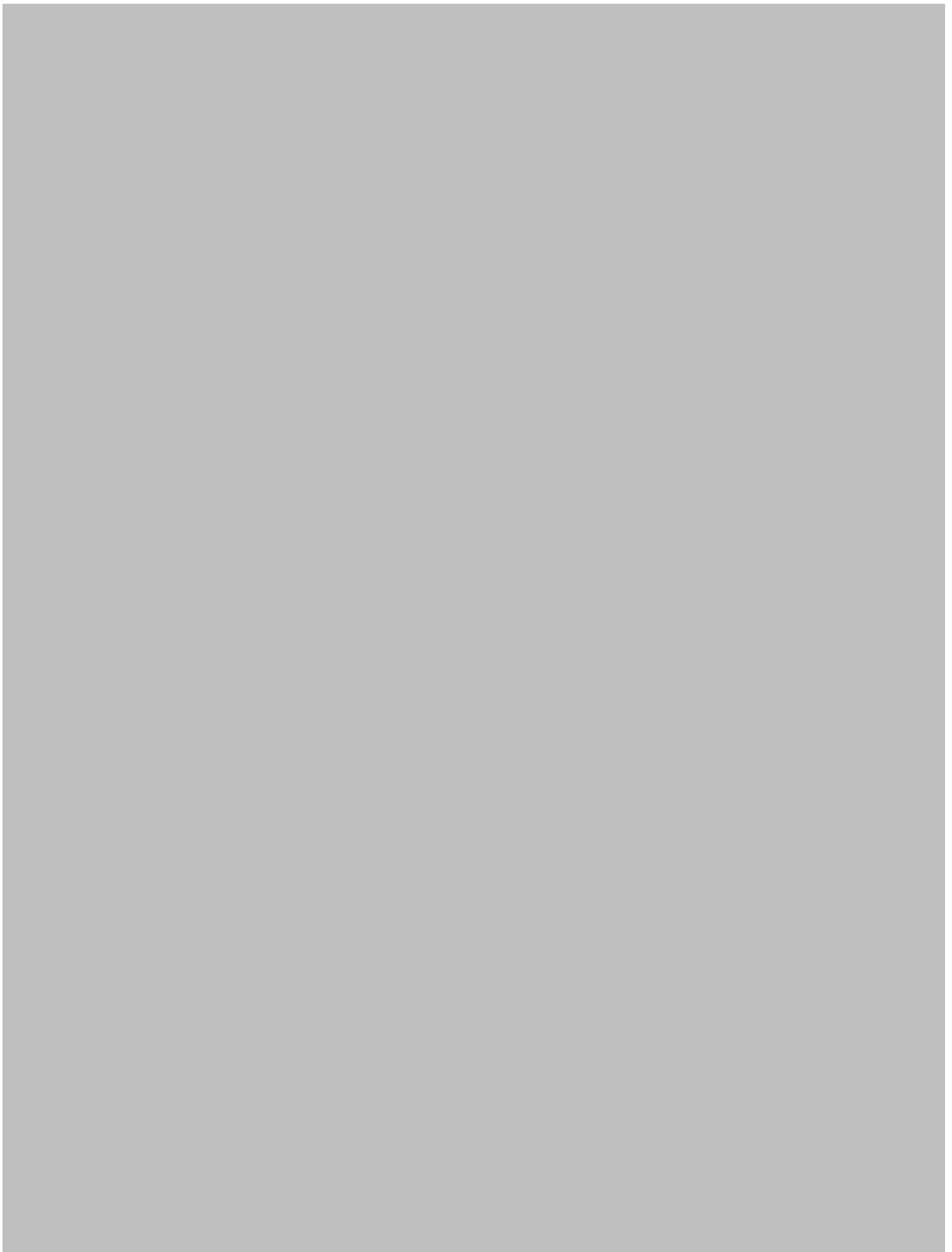
The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that proper record-keeping is essential for the success of any business and for the protection of the interests of all parties involved. The document then goes on to describe the various methods and techniques used to collect and analyze data, highlighting the importance of using reliable sources and ensuring the accuracy of the information collected.

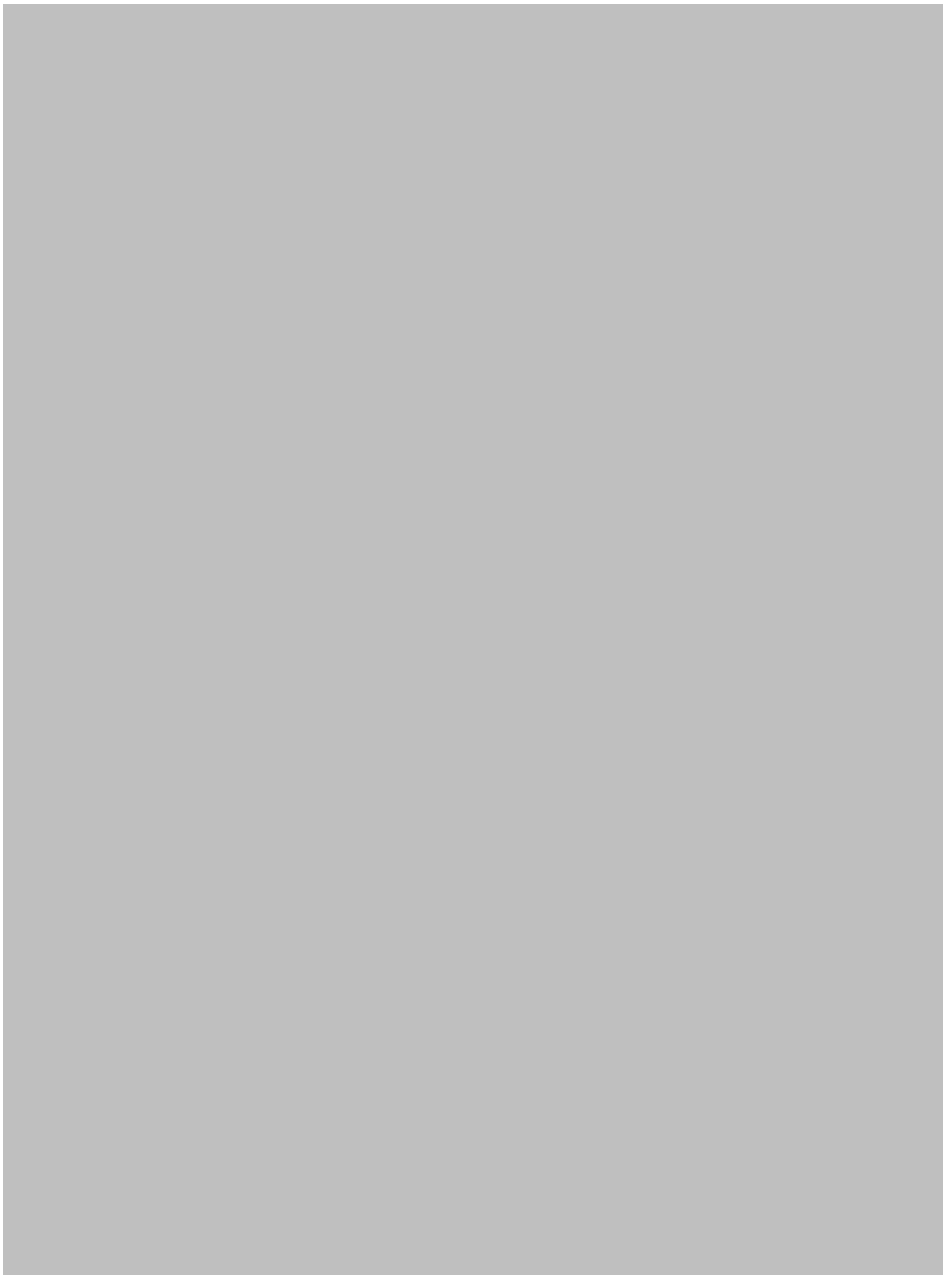
The second part of the document focuses on the analysis of the data collected. It discusses the various statistical methods and techniques used to analyze the data, including the use of regression analysis, correlation analysis, and other advanced statistical techniques. The document also discusses the importance of interpreting the results of the analysis and drawing meaningful conclusions from the data.

The third part of the document discusses the application of the results of the analysis. It describes the various ways in which the results can be used to inform decision-making and to improve the performance of the business. The document also discusses the importance of communicating the results of the analysis to the relevant stakeholders and ensuring that they are understood and acted upon.

The final part of the document discusses the future of data analysis and the role of technology in this field. It describes the various emerging technologies and techniques that are being used to improve the accuracy and efficiency of data analysis, and discusses the potential for these technologies to revolutionize the way in which businesses collect and analyze data.









## CHAPTER 3

### PUBLICATION 2: REVIEW

**Duncan HF, Smith AJ, Fleming GJP, Cooper PR. (2016). Epigenetic modulation of dental pulp stem cells: implications for regenerative endodontics. *Int Endod J* doi: 10.1111/iej.12475. [Epub ahead of print]**

During the course of these studies, it was evident that epigenetic modifying agents and HDACi in particular, could exert differing effects on a range of cell types. Primary cells were generally more resistant to the effects of HDACis than transformed cells, while notably stem cell (SC) populations reacted differently from adult differentiated cell populations. Critically, it became evident that SC commitment, in either self-renewal or differentiation, was controlled at least in part by epigenetic signalling and marks. As a result, the current knowledge of HDACi, and other epigenetic-modifying agents, in altering SC differentiation, de-differentiation and reprogramming was reviewed in the context of the development of novel regenerative therapies for the dentine-pulp complex.

The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every receipt, invoice, and bill should be properly filed and dated. This not only helps in tracking expenses but also provides a clear audit trail for tax purposes. The text suggests using a systematic approach, such as the envelope system, to ensure that no financial activity goes unrecorded.

Next, the document addresses the issue of budgeting. It argues that a well-defined budget is essential for controlling spending and achieving financial goals. By allocating funds to specific categories, individuals can avoid unnecessary purchases and ensure that their income covers all necessary expenses. The text provides several tips for creating a realistic budget, including tracking current spending patterns and setting aside a contingency fund for unexpected costs.

The third section focuses on debt management. It highlights the dangers of accumulating high-interest debt and offers strategies for reducing and eliminating it. Key advice includes prioritizing payments on high-interest loans, negotiating with creditors for better terms, and considering debt consolidation options. The document stresses that staying on top of payments and avoiding late fees are crucial for maintaining a healthy credit profile.

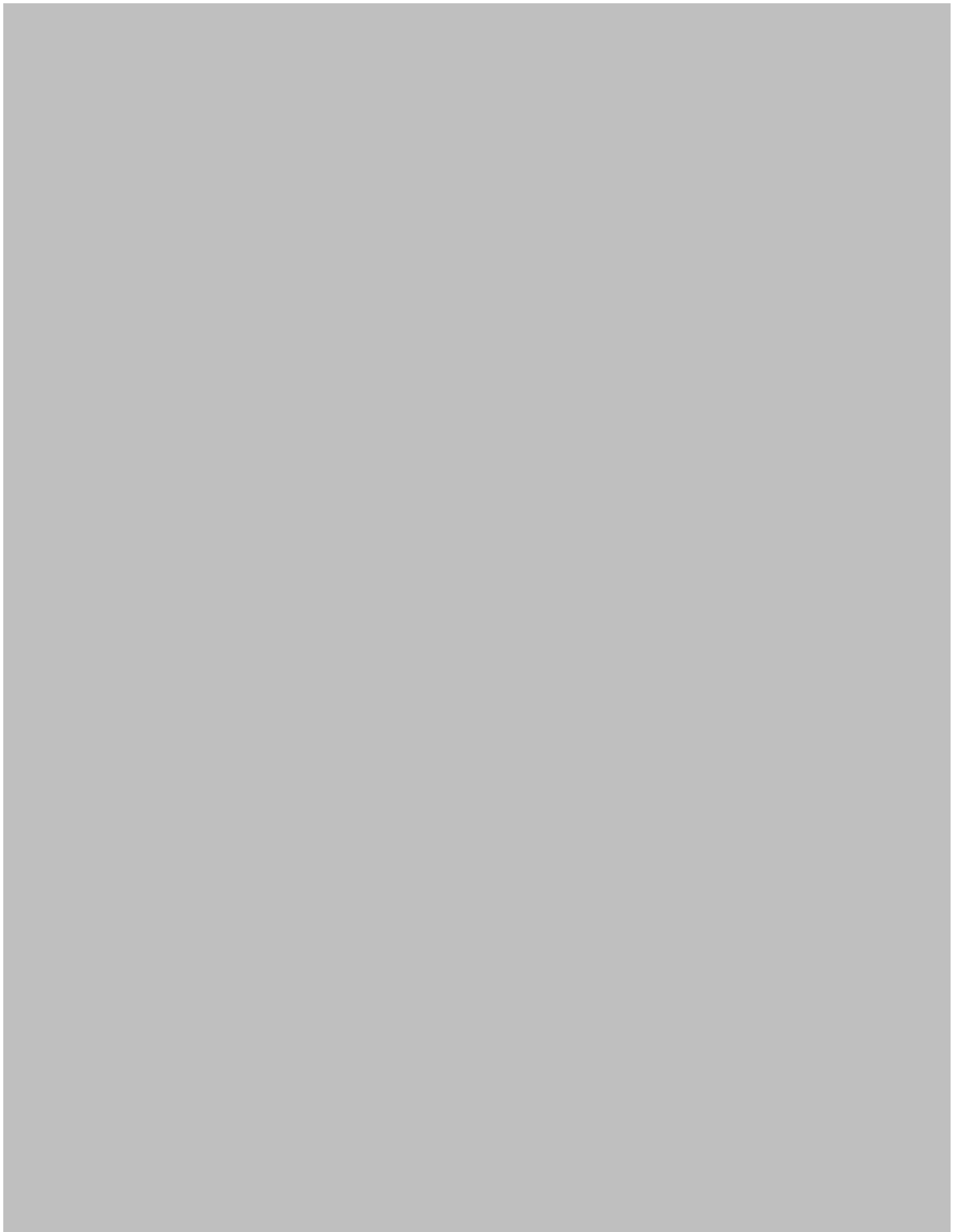
Finally, the document touches upon investment and long-term financial planning. It encourages individuals to start saving early and take advantage of tax-advantaged investment vehicles like 401(k) plans and IRAs. The text explains the power of compound interest and how consistent contributions over time can significantly grow wealth. It also mentions the importance of diversifying investments to manage risk and seeking professional advice when needed.

The first part of the document discusses the importance of maintaining accurate records in a business setting. It highlights how proper record-keeping can help in decision-making, legal compliance, and financial management. The text emphasizes that records should be organized, up-to-date, and easily accessible to all relevant personnel.

Next, the document addresses the challenges of data management in the digital age. With the increasing volume of data generated by various systems, businesses face the task of storing, securing, and analyzing this information effectively. The text suggests implementing robust data management strategies, including regular backups, security protocols, and the use of data analytics tools to extract meaningful insights from the data.

The third section focuses on the role of technology in streamlining business operations. It explores how automation and digital tools can reduce manual tasks, improve efficiency, and minimize errors. Examples include using accounting software for financial tracking, project management tools for task delegation, and CRM systems for customer relationship management. The text encourages businesses to invest in technology that aligns with their specific needs and goals.

Finally, the document discusses the importance of employee training and development. In a rapidly changing business environment, it is crucial for employees to have the necessary skills and knowledge to perform their roles effectively. The text suggests implementing a comprehensive training program that covers both technical and soft skills, and encourages a culture of continuous learning and professional growth.

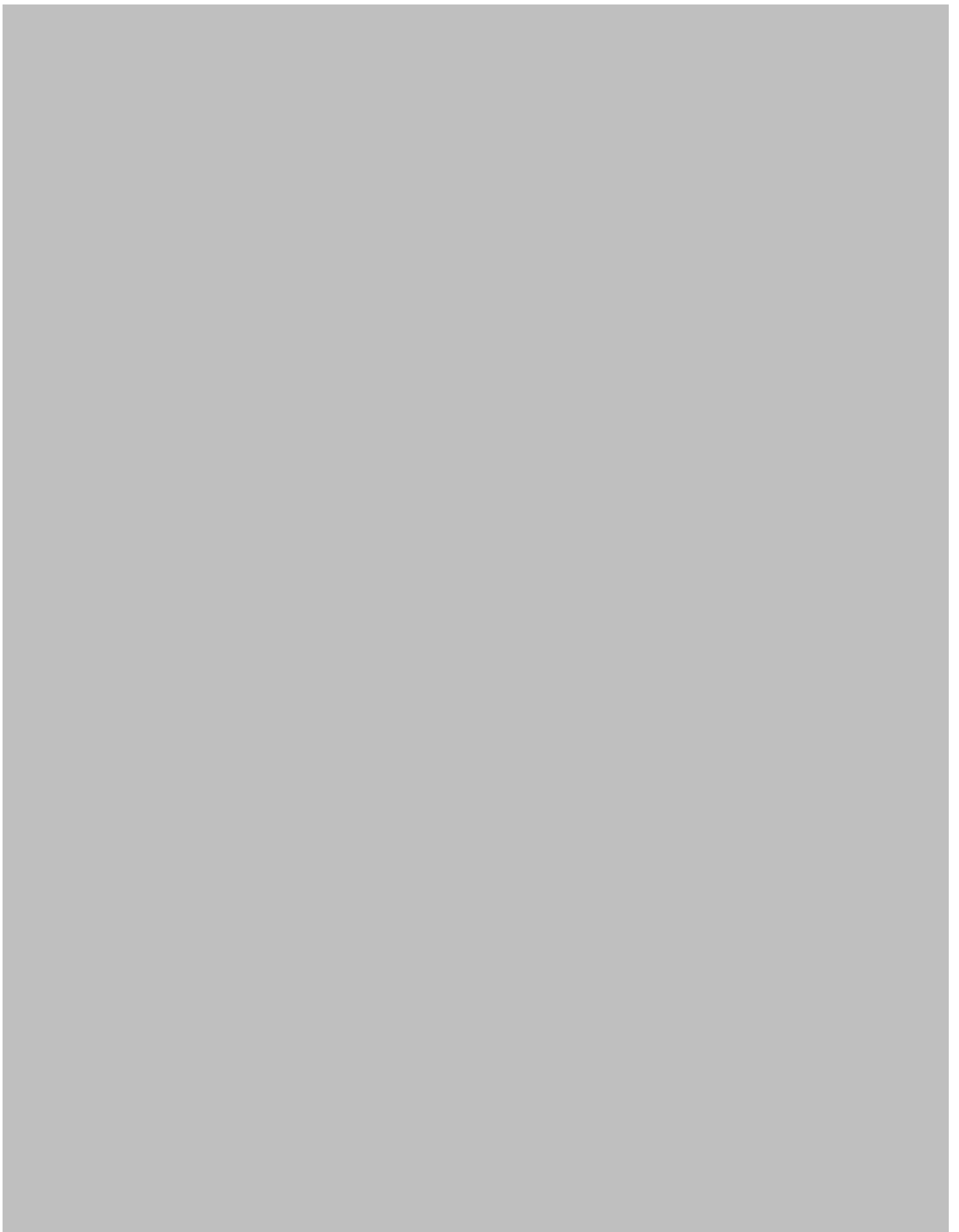


The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every entry, no matter how small, should be recorded to ensure the integrity of the financial data. This includes not only sales and purchases but also expenses and income. The text explains that proper record-keeping is essential for identifying trends, managing cash flow, and preparing for tax obligations. It also notes that clear records can help in resolving any disputes or discrepancies that may arise.

The second section focuses on the role of the accounting system in providing a clear and concise overview of the company's financial health. It describes how a well-designed system can automate many of the routine tasks, reducing the risk of human error and saving valuable time. The text highlights the importance of regular reviews and reconciliations to ensure that the books are balanced and that all transactions are properly accounted for. It also discusses the benefits of using modern accounting software, which can provide real-time insights into the company's performance and help in making informed decisions.

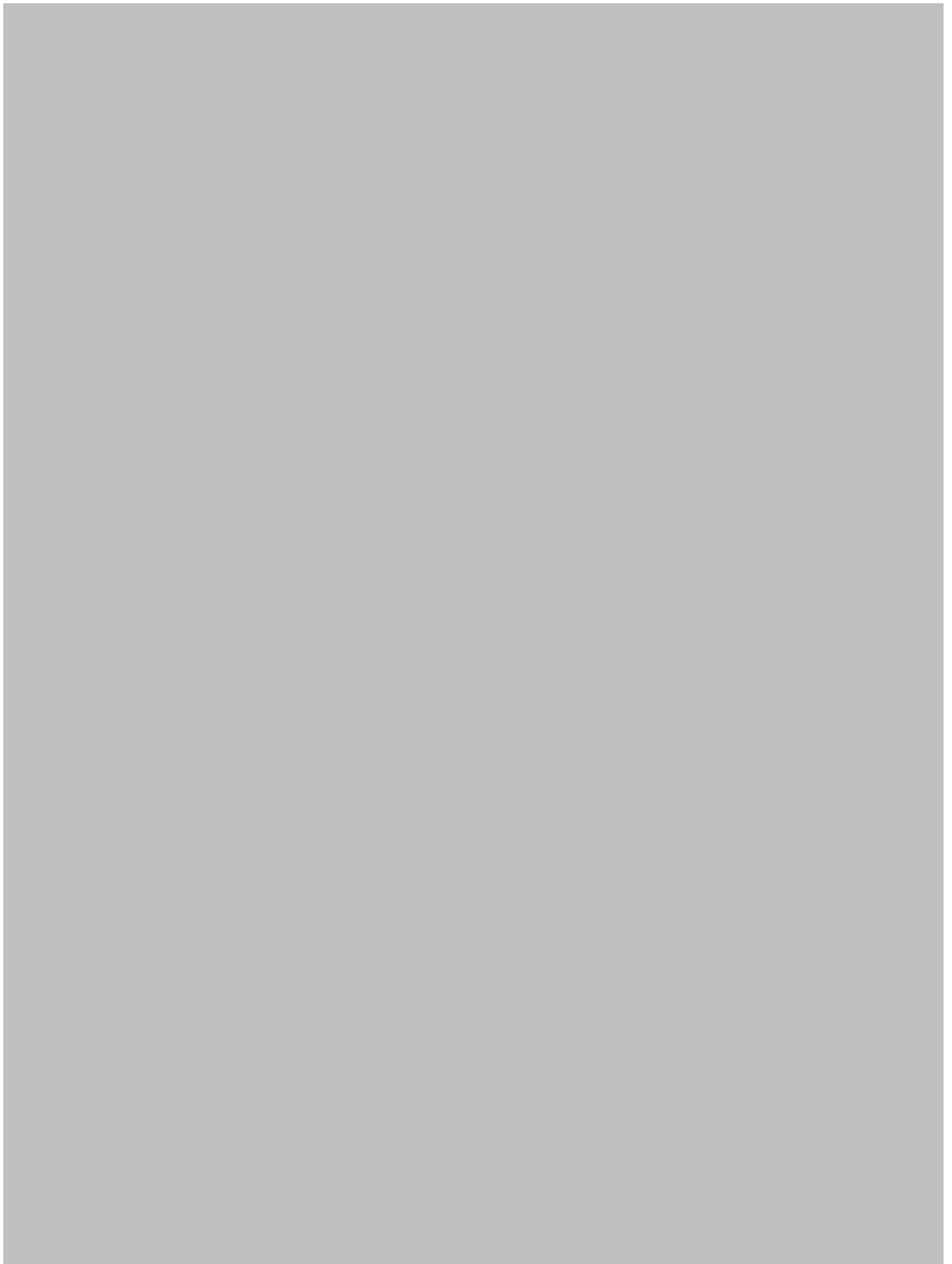
The final part of the document addresses the challenges of managing financial data in a complex and ever-changing business environment. It acknowledges that there are many factors that can affect the accuracy and reliability of the data, such as changes in accounting standards, new regulations, and technological advancements. The text offers practical advice on how to stay up-to-date with the latest developments and how to implement effective controls to mitigate the risks. It concludes by emphasizing that a strong financial foundation is crucial for the long-term success and sustainability of any business.

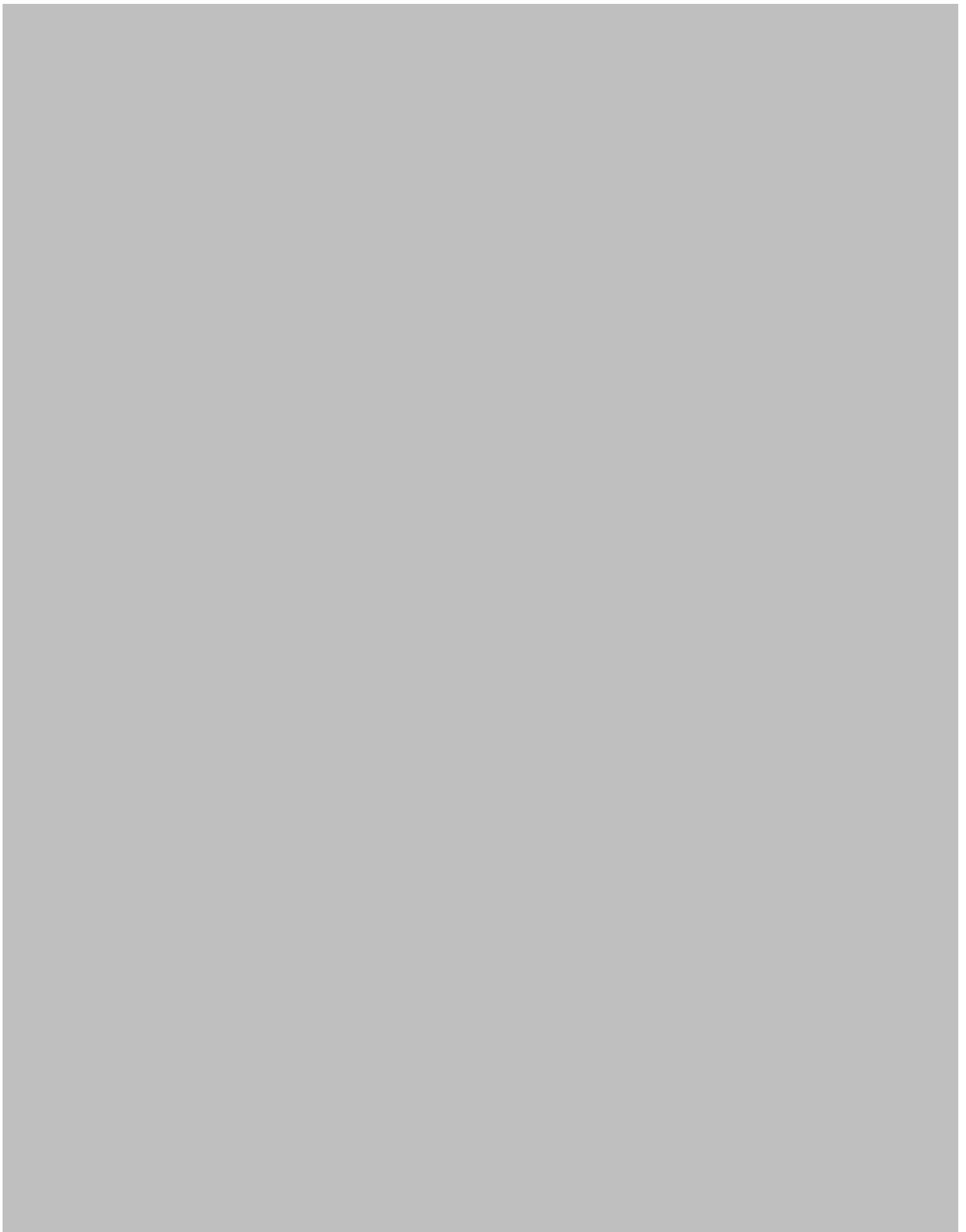


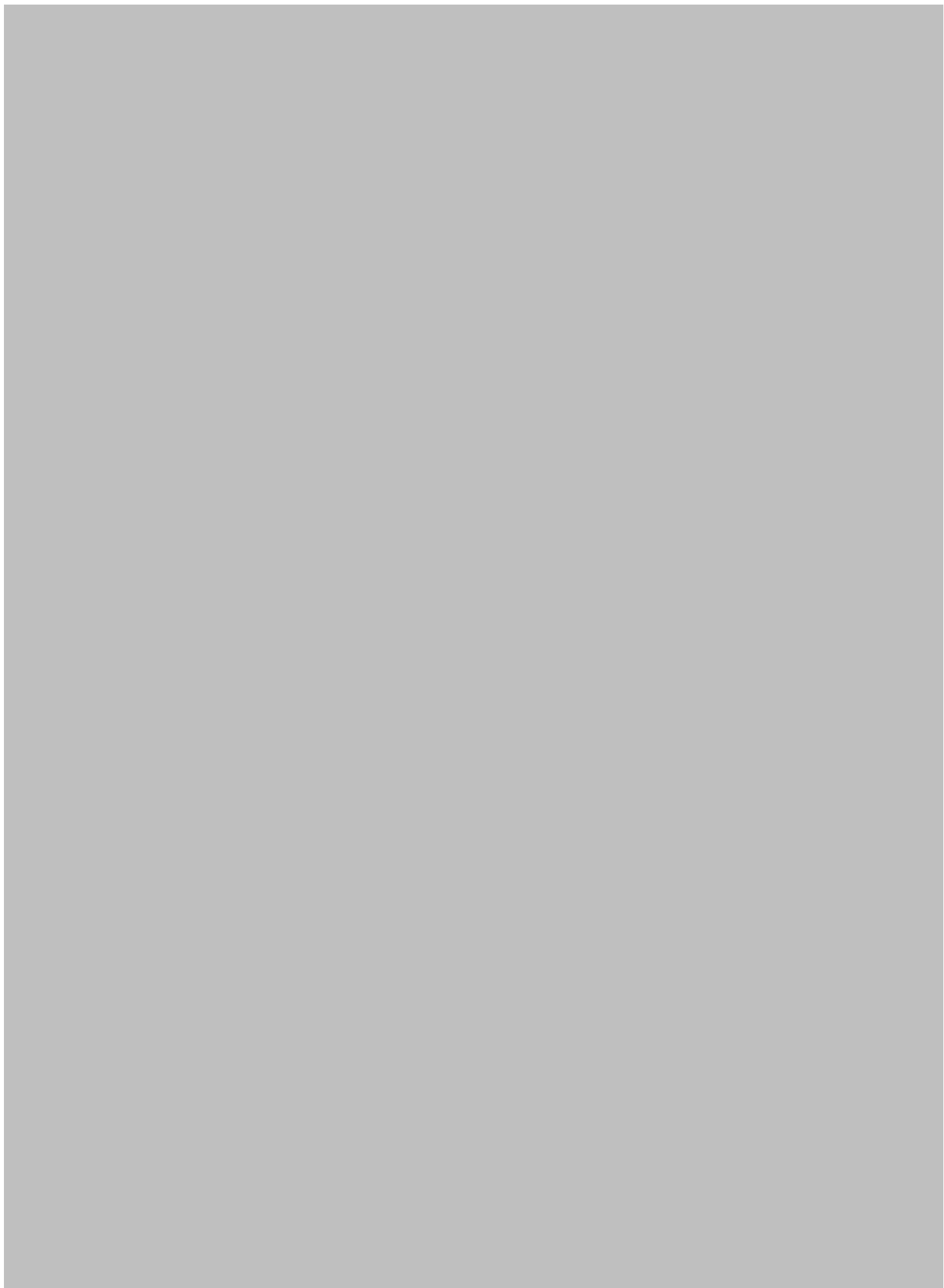


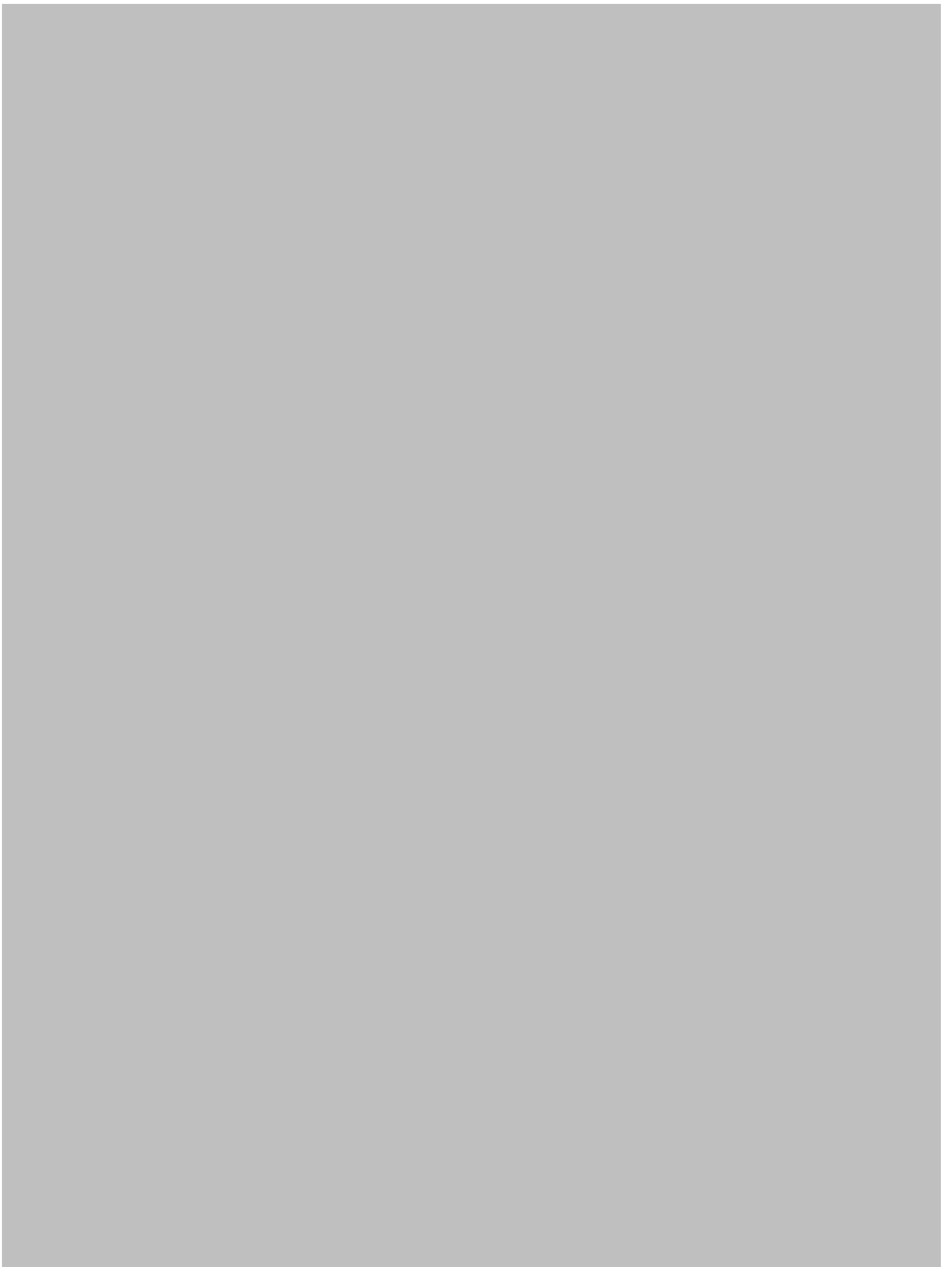


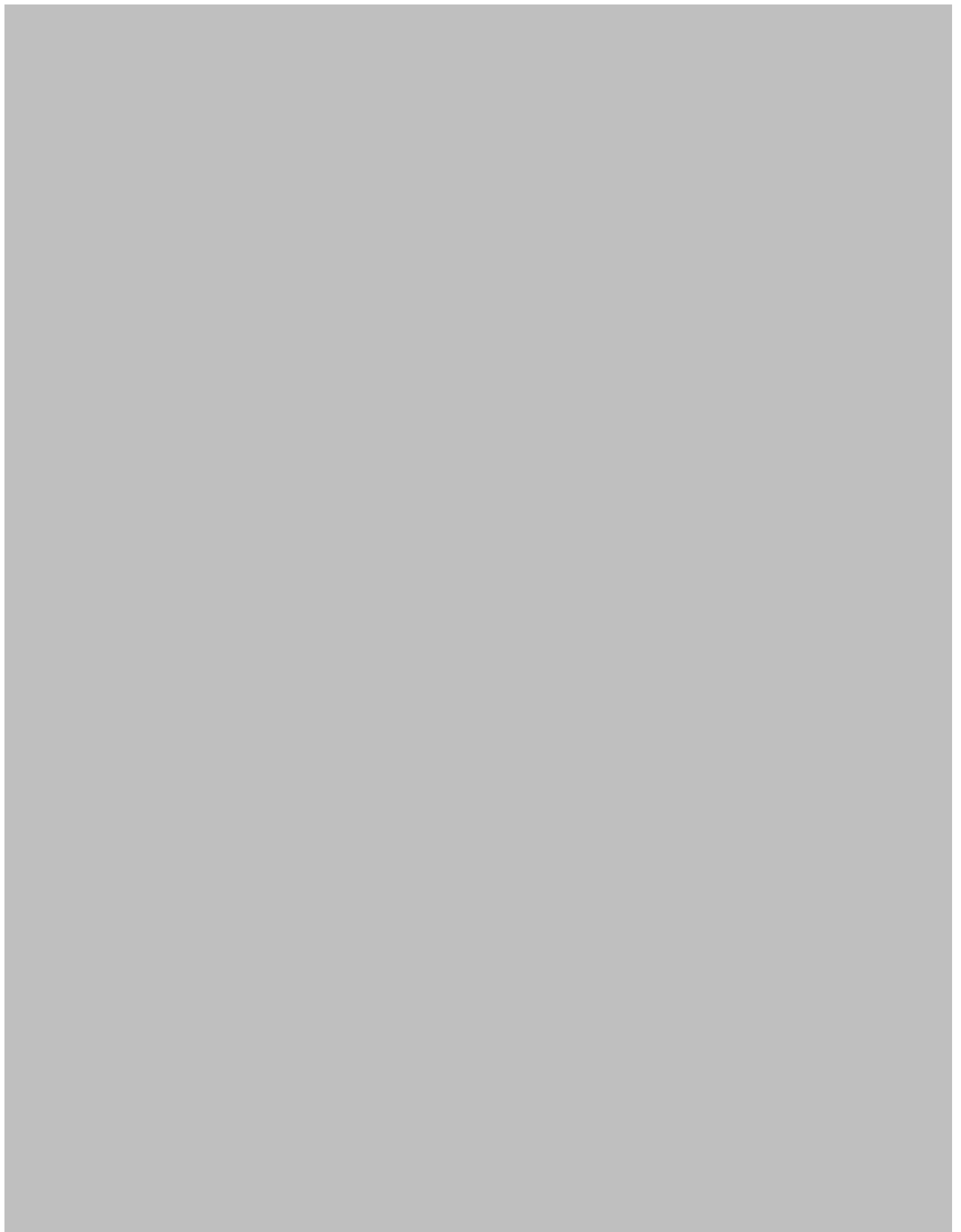


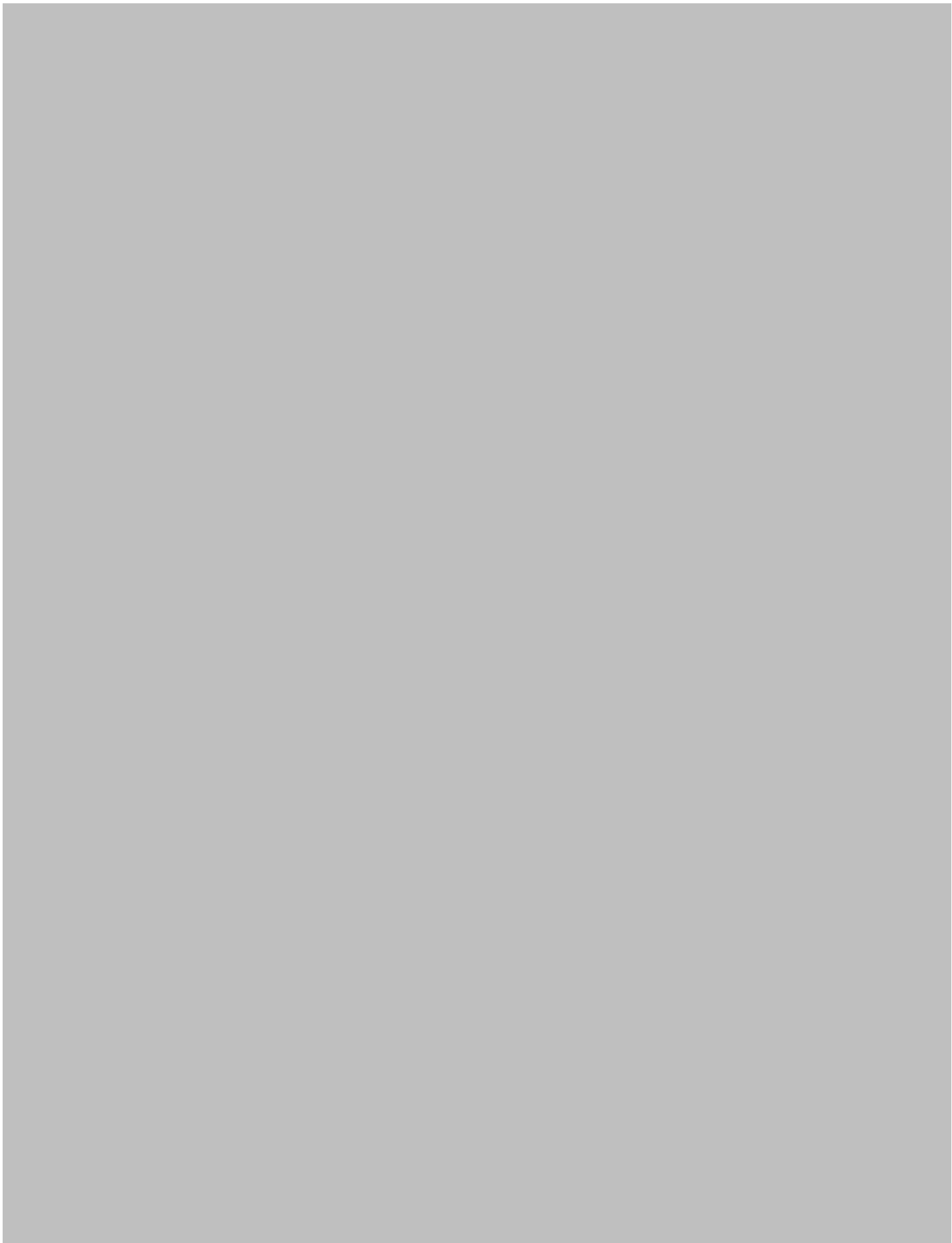








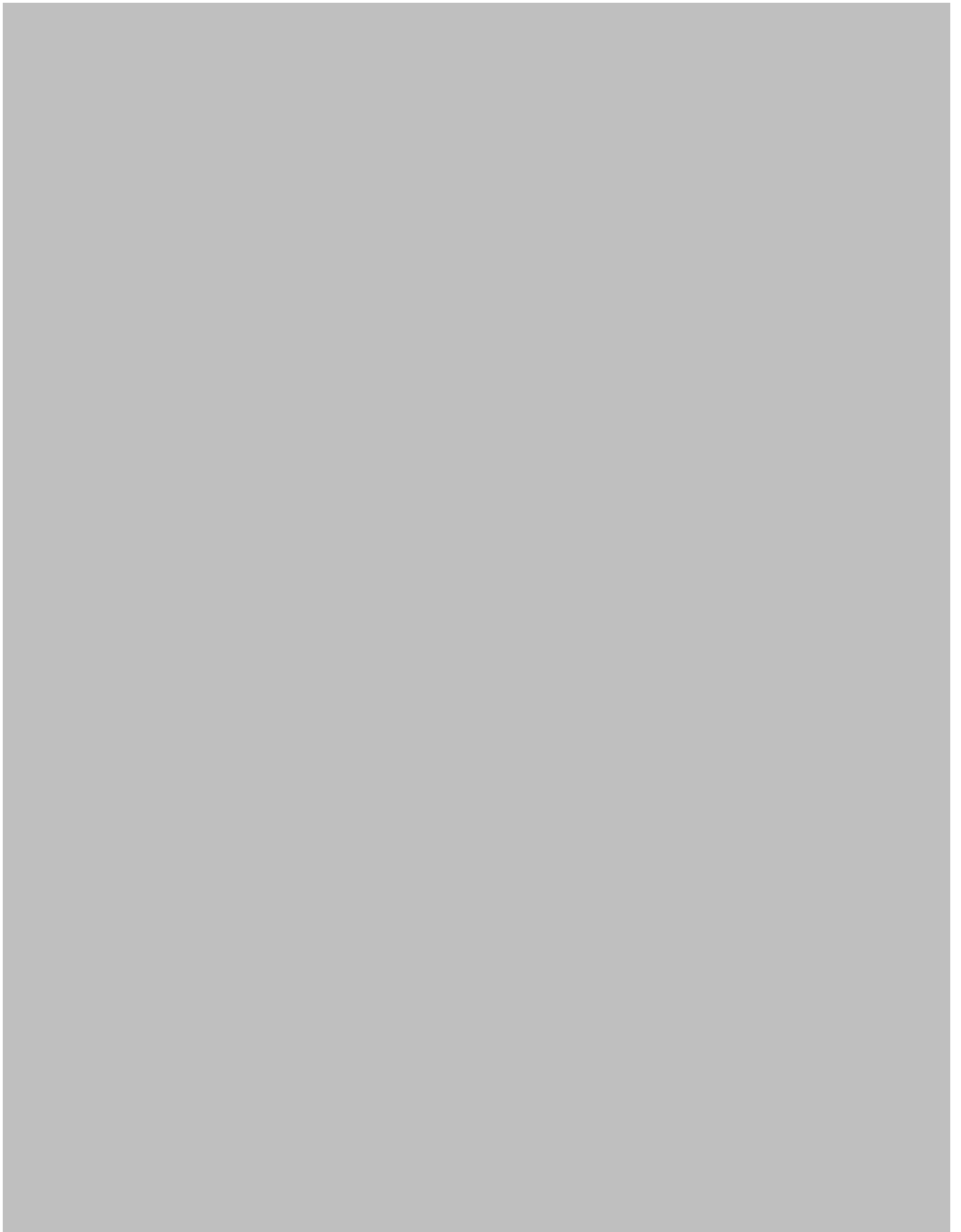




The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that proper record-keeping is essential for the success of any business and for the protection of the interests of all parties involved. The document then goes on to describe the various methods and techniques used to collect and analyze data, highlighting the importance of using reliable sources and ensuring the accuracy of the information gathered.

The second part of the document focuses on the analysis of the data collected. It discusses the various statistical methods and techniques used to interpret the results, including the use of regression analysis, correlation analysis, and hypothesis testing. The document also discusses the importance of understanding the limitations of the data and the potential sources of error, and provides guidance on how to address these issues.

The final part of the document discusses the implications of the findings and provides recommendations for future research. It emphasizes the need for continued research in this area and the importance of staying up-to-date on the latest developments in the field. The document concludes by reiterating the importance of accurate record-keeping and the need for ongoing research to improve our understanding of the various factors that influence business success.







## CHAPTER 4

### PUBLICATION 3: ORIGINAL SCIENTIFIC MANUSCRIPT

**Duncan HF, Smith AJ, Fleming GJP, Cooper PR. (2012). Histone deacetylase inhibitors induced differentiation and accelerated mineralization of pulp-derived cells. *J Endod* 38:339-345.**

<http://dx.doi.org/10.1016/j.joen.2011.12.014>

Based on the previous review of HDACi-induced cellular effects (Chapter 2), it was hypothesised that HDACis would positively affect cell proliferation, promote differentiation and accelerate mineralisation events in a dental papillae-derived cell line (MDPC-23). The experimental methodologies were specifically designed to investigate the cellular effects of two pan-HDACis (TSA and VPA), applied to cell cultures at a range of relatively low concentrations. Cell viability was assessed by cell counting and trypan blue exclusion, cell cycle and apoptosis were analysed by immunocytochemical staining and high content analyses, while cellular mineralisation responses were investigated by quantitative Alizarin red staining and reverse transcriptase polymerase chain reaction (RT-PCR).

# Histone Deacetylase Inhibitors Induced Differentiation and Accelerated Mineralization of Pulp-derived Cells

Henry F. Duncan, BDS, MClin Dent,\* Anthony J. Smith, PhD,<sup>‡</sup> Garry J.P. Fleming, PhD,<sup>†</sup> and Paul R. Cooper, PhD<sup>‡</sup>

## Abstract

**Introduction:** Histone deacetylase inhibitors (HDACis) alter the homeostatic balance between 2 groups of cellular enzymes, histone deacetylases (HDACs) and histone acetyltransferases (HATs), increasing transcription and influencing cell behavior. This study investigated the potential of 2 HDACis, valproic acid (VPA) and trichostatin A (TSA), to promote reparative processes in pulp cells as assayed by viability, cell cycle, and mineralization analyses. **Methods:** VPA (0.125–5 mmol/L) and TSA (12.5–400 nmol/L) were applied to a pulp-derived cell population and compared with un-supplemented controls. Cell proliferation and viability were evaluated by trypan blue staining and cell counting, whereas cell cycle and apoptosis were analyzed by immunocytochemical staining with antibodies for p53, phosphorylated p53, Bcl-2 homologous antagonist/killer (BAK), caspase-3 and p21<sup>WAF1/CIP</sup>, and DNA staining with Hoechst 33342. For mineralization analysis, cultures were stained with Alizarin red and quantified spectrophotometrically. Relative gene expression levels of mineralization associated markers were analyzed using reverse-transcriptase polymerase chain reaction. One-way analysis of variance and Tukey post hoc tests were applied to the data ( $P < .05$ ). **Results:** VPA and TSA reduced cell proliferation dose dependently with no significant effect on cell viability except at 400 nmol/L TSA. The transcription factor p21<sup>WAF1/CIP</sup> was significantly increased at the highest concentration of TSA but not VPA. Significant increases ( $P < .05$ ) in the apoptosis marker protein active caspase-3 and cell cycle alterations were only evident at the maximum concentrations of TSA/VPA, whereas HDACi-induced mineralization per cell was stimulated dose dependently with a significant increase in the expression of the dentinogenic-associated transcript, dentine matrix protein-1. **Conclusions:** These results indicate that HDACis are capable of epigenetically modulating pulp cell behavior, signifying their therapeutic potential for

augmenting biomaterials, and stimulating regenerative responses in the damaged pulp. (*J Endod* 2012;38:339–345)

## Key Words

Dental pulp cells, dental restorative material, epigenetics, histone deacetylase inhibitors, regeneration

Exciting opportunities exist for the development of a new generation of dental materials that are functionally modified to act on various biological processes thereby stimulating regenerative events (1, 2). Such materials may target tissue injury-associated events using antioxidants (N-acetyl cysteine) (2–4) or may directly stimulate regeneration by the addition of growth factors (5–7). However, targeting cell nuclear function offers a novel mode of action because acetylation of DNA-associated histone and non-histone proteins influences gene expression and induces various cellular processes (8–10). Acetylation is regulated by the cellular enzymes, histone acetyltransferases (HATs), and histone deacetylases (HDACs) (11). HAT activity relaxes the structure of chromatin rendering it transcriptionally active, thereby increasing gene expression, whereas HDAC represses transcription (12).

Histone deacetylase inhibitors (HDACis) modify the balance between HATs and HDACs, leading to the accumulation of acetylated proteins with associated transcriptional and cellular effects, thereby influencing proliferation, differentiation, and apoptosis (11, 13). A host of natural and synthetic HDACis exist, including valproic acid (VPA) and trichostatin A (TSA), which have similar nuclear action but differ in chemical structure and selectivity (13–15). Although originally investigated as prodifferentiation adjuncts in the treatment of cancer cells (11, 13), HDACi-mediated effects also provide novel therapeutic approaches in normal cells and are currently being investigated for application in bone engineering (16), inflammatory diseases (17), and stem cell engineering (18). HDACis alter cell phenotype by a range of mechanisms, including modulation of gene expression and intracellular signaling, indicating a potential therapeutic role in promoting reparative processes in pulp cells (10). Specifically, the application of VPA, TSA, and other HDACis increases *in vitro* expression of osteogenic-related markers in primary cells and cell lines, including alkaline phosphatase (16, 19), collagen-1 $\alpha$ , osteocalcin, osteopontin, and bone sialoprotein (20, 21). Concomitantly, HDACis accelerate osteoblast differentiation and mineralization processes (16, 20, 21). TSA has been shown to epigenetically up-regulate stem cell markers in regenerative amputation sites (22). VPA and other HDACis have been shown to reduce circulating proinflammatory cytokine production (15, 17) and further control inflammatory processes by

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modulating the transcriptional accessibility of the nuclear factor-kappa B (NF- $\kappa$ B) gene (23).

The promotion of tissue regeneration after dental pulp damage resulting in tertiary dentinogenesis is a key goal of vital pulp therapy (5). HDACis have not yet been investigated within restorative dentistry but could potentially address current vital pulp treatment limitations associated with inflammation (24) and unpredictable mineral deposition (25). Their incorporation or adjunctive use with dental restorative approaches may subsequently facilitate the development of more targeted “regenerative restorative biomaterials” (1, 2), which is similar to previous work that has included antibacterials and antioxidants (26, 27). Based on previous studies, we hypothesized that a synthetic and natural HDACi, VPA (15), and TSA (14) could positively affect cell proliferation, cell differentiation, and cell cycle, leading to accelerated mineralization events in dental pulp-derived cells.

### Materials and Methods

#### HDACis

A stock 1 mol/L VPA (2-propylpentanoic acid sodium salt) (Sigma-Aldrich, Arklow, Ireland) in phosphate-buffered saline (PBS) was diluted with supplemented cell culture medium; 5 mmol/L TSA (7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamidine) (Sigma-Aldrich) in dimethyl sulfoxide was diluted in PBS before supplementation in culture medium.

#### Cell Culture

The murine dental pulp-derived cell line (MDPC-23) was used (28). Cells were grown in high glucose (4.5 g/L) Dulbecco modified Eagle medium (DMEM) (Biosera, East Sussex, UK) supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin (10,000 U/mL penicillin with 10,000  $\mu$ g/mL streptomycin), and 10% (v/v) fetal calf serum (Biosera) at 95% relative humidity in a 5% CO<sub>2</sub> atmosphere at 37°C.

#### Evaluation of Cell Proliferation and Cell Viability

Cells were seeded ( $5 \times 10^4$ ) on 6-well culture dishes (Sarstedt, Wexford, Ireland) and cultured in 2 mL supplemented DMEM. At 24 hours, VPA (0.25 mmol/L, 0.5 mmol/L, 1 mmol/L, 3 mmol/L, and 5 mmol/L) and TSA (12.5 nmol/L, 25 nmol/L, 50 nmol/L, 100 nmol/L, 200 nmol/L, and 400 nmol/L) were supplemented in cultures and incubated for a further 72 hours. The positive control samples contained cells cultured in DMEM not supplemented with HDACis, whereas the negative control samples contained no cells. Cells were detached with Hank's balanced salt solution containing 2.5 g/L trypsin and 0.2 g/L EDTA and stained with 0.4% trypan blue before cell counting and viability assessment using a standard hemocytometer. Cell proliferation for each experimental HDACi concentration was calculated and charted as % control.

#### Cell Cycle and Apoptosis

To determine the influence of HDACi on cell cycle and apoptosis, high-content analysis (HCA) was performed. Cells were seeded ( $4 \times 10^3$ ) in 96-well plates (Sigma-Aldrich, Arklow, Ireland) for 24 hours before culture in supplemented DMEM containing TSA (50 nmol/L, 100 nmol/L, 200 nmol/L, and 400 nmol/L) or VPA (0.5 mmol/L, 1 mmol/L, 3 mmol/L, and 5 mmol/L) for 24 and 48 hours. Positive controls contained cell cultures not supplemented with HDACi (0 nmol/L/mmol/L concentration); negative controls contained no cells. Cells were fixed in 1% formaldehyde in PBS for 30 minutes and processed (Imagen Biotech, Manchester, UK) by permeabilization and stained with antibodies (diluted to 500  $\mu$ g/mL in digitonin/PBS

solutions), anti-BAK (Cat# AM03; Merck, Darmstadt, Germany), active caspase 3 (Cat# ab13847; Abcam, Cambridge, UK), p21<sup>WAF1/CIP</sup> (Cat# PN8400602; Thermo Fisher Scientific, Leicestershire, UK), p53 (Cat# PN8400602, Thermo Fisher Scientific), and phosphorylated p53 (Cat# PN8400702, Thermo Fisher Scientific). Immunoreactions were visualized using 2 fluorophore-conjugated secondary antibodies, goat antimouse 488, and goat antirabbit 594 (Cat# 11001 and 11037 respectively; Invitrogen, Paisley, UK). For cell cycle analysis, DNA was stained with Hoechst 33342 at 2.5  $\mu$ g/mL, and the % of each component of the cell cycle was recorded (Invitrogen). Cultures were analyzed on an Arrayscan using a compartmental analysis algorithm (Cellomics, Thermo Fisher Scientific). The number of cells expressing the marker protein was recorded as a percent of the total number of cells scanned.

#### Mineralization Assay

Cells were seeded ( $5 \times 10^4$ ) on 6-well culture dishes and cultured in 2 mL supplemented DMEM for 72 hours until 60% to 70% confluent before exposure to HDACi-supplemented mineralizing medium (DMEM supplemented with 50  $\mu$ g/mL ascorbic acid, 0.1  $\mu$ mol/L dexamethasone, and 10 mmol/L  $\beta$ -glycerophosphate  $\pm$  VPA or TSA concentrations) for the duration of the experiment. Cultures were incubated for 1, 4, 7, and 10 days with regular mineralizing media changes. For mineralization analysis, 10-day cultures were washed  $3 \times 5$  minutes in PBS and fixed in 10% formaldehyde for 15 minutes, washed with distilled water, and then stained with 1.37% (w/v) Alizarin red S (Millipore, Cork, Ireland) (pH = 4.2) for 15 minutes at room temperature. The stain was solubilized and quantified spectrophotometrically at 405 nm (29). For the time-course study, cell numbers were determined at 1, 4, 7, and 10 days (as for proliferation and viability studies); after detachment, trypan blue staining and standard counting using a hemocytometer were performed. Mineral production per cell data for VPA and TSA were based on quantification of Alizarin red S stain (in mmol/L) extracted from each experimental well and the corresponding 10 day time-course data for each HDACi.

#### Reverse-transcriptase Polymerase Chain Reaction

Cells were seeded ( $5 \times 10^4$ ) onto a 6-well culture dish and 2 mL supplemented mineralizing medium containing 1 mmol/L VPA or 100 nmol/L TSA were added after 24 hours. HDACi concentrations used were determined from the optimum results of the proliferation, viability, cell cycle, and mineralization studies. Positive controls contained no HDACis, whereas negative controls were cultured only in supplemented DMEM. Cultures were incubated for a further 72 hours, detached with trypsin/EDTA, homogenized for 30 seconds using a T10 basic S2-Ultra-Turrax tissue disrupter (IKA, Staufen, Germany), and RNA extracted using the RNeasy mini kit (Qiagen, West Sussex, UK) before elution in 30  $\mu$ L of sterile water. RNA concentrations were determined spectrophotometrically at 260 nm, and RNA was converted into single-stranded complementary DNA using the Omniscript kit (Qiagen) and oligo-dT primer (Ambion; Applied Biosciences, Dublin, Ireland). Synthesized complementary DNA was concentrated and purified by centrifugation with Microcon YM-30 filters (Millipore, Cork, Ireland) and complementary DNA concentrations determined spectrophotometrically.

Semi-quantitative reverse-transcriptase polymerase chain reaction analysis was performed for mouse genes using the following specific primers: dentine matrix phosphoprotein-1 (DMP-1) (F)-5' CCCATCACCATCTTCCAGGAGC 3'/(R)-5' CCAGTGAGCTTCCCGTTCAGC, bone morphogenic protein-4 (BMP-4) (F)-5' CAGGGCTTCCACCGTA-TAAA/ (R)-5' ATGCTTGGGACTACGTTTGG, and transforming growth factor beta-1 (TGF- $\beta$ -1) (F)-5' CTGTCCAACTAAGGCTCGC/ (R)-5' CGTCAAAGACAGCCACTCA normalized to glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) (F)-5' CCCATCACCATCTTCCAGGAGC/ (R)-5' CCAGTGAGCTTCCCGTTCAGC (Invitrogen). Each polymerase chain reaction included 12.5  $\mu$ L REDTaq ReadyMix Polymerase Chain Reaction Mix (with MgCl<sub>2</sub>) (Sigma-Aldrich), 0.5  $\mu$ L forward/reverse primer, 10  $\mu$ L molecular grade water, and 50 ng of template complementary DNA. Polymerase chain reactions were performed using the GS-1 G-Storm thermal cycler (G-Storm, Surrey, UK) and subjected to the designated number (20-45) of amplification cycles in which a typical cycle was performed at 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. After thermal cycling, amplified products were electrophoresed on a 1.5% agarose gel supplemented with gel red (Biotium, Hayward, CA) and visualized under ultraviolet light with the AlphaImager-Mini system (Alpha Innotech/Cell Biosciences, Santa Clara, CA). Captured gel images were imported into AlphaView image analysis software (Alpha Innotech/Cell Biosciences) for densitometric analysis. The volume density of amplified products was determined and normalized to GAPDH intensity levels. The averaged values for each group were calculated and expressed as a percentage of the highest normalized individual band density obtained for each DMP-1, BMP-4, and TGF- $\beta$ 1 transcript.

## Statistical Analysis

One-way analyses of variance and Tukey post hoc tests were used ( $P < .05$ ) for cell proliferation, cell viability, cell cycle, apoptosis markers, mineralization per cell data, and reverse-transcriptase polymerase chain reaction to determine the influence of HDACi concentration on the cells using SigmaStat 12.0.1 software (SPSS Inc, Chicago, IL). Linear regression analyses were applied to the mineralization per cell data for the examination of the dose-dependent effects of VPA and TSA.

## Results

### Cell Proliferation and Cell Viability

Cell proliferation was significantly inhibited by treatment for 3 days with VPA ( $P < .003$ ) and TSA ( $P < .001$ ) compared with control unstimulated cultures at all concentrations, with the exception of 0.25 mmol/L VPA ( $P = .152$ ) and 12.5 nmol/L TSA ( $P = .066$ ) (Fig. 1A and B). Cell viability (trypan blue exclusion) was not significantly reduced with VPA ( $P > .299$ ) or TSA ( $P > .188$ ) with the exception of 400 nmol/L TSA ( $P = .011$ ) (Fig. 1A and B) when compared with the control.

### Cell Cycle and Apoptosis

At 24 and 48 hours, there were no significant changes in nuclear p53 and BAK levels with VPA and TSA (all  $P > .05$ ) compared with

untreated cells (data not shown). Phosphorylated p53 showed a significant increase in expression at 24 hours ( $P < .0001$ ) but not at 48 hours ( $P > .054$ ) for VPA (Fig. 2A). No significant changes in expression at 24 hours ( $P > .059$ ) or 48 hours (all  $P > .816$ ) for TSA were evident (Fig. 2B). No difference in transcription factor p21 expression was shown at 24 hours ( $P > .999$ ) or 48 hours ( $P > .927$ ) for VPA or 48 hours for TSA ( $P = 1.0$ ) (Fig. 2C and D). A significant increase in p21 expression at 24 hours was identified at 400 nmol/L TSA ( $P < .0001$ ) and a significant increase in active caspase-3 was observed with 5 mmol/L VPA at 24 hours ( $P = .017$ ) (Fig. 2E) and 400 nmol/L TSA at 24 hours ( $P = .038$ ) and 48 hours ( $P < .0001$ ) (Fig. 2F). Cell cycle analysis at 48 hours indicated minimal alteration with HDACis ( $P > .124$ ) although there was a decrease in cells in the G2 phase and G2/S after the administration of 5 mmol/L VPA ( $P = .003$ ) (Fig. 2G) and 400 nmol/L TSA ( $P = .002/.013$ ), respectively (Fig. 2H).

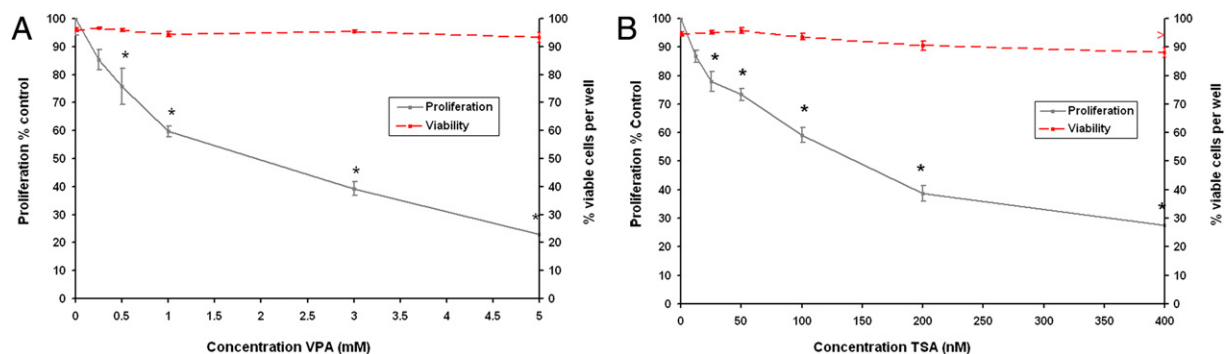
## Mineralization

Quantitative measurement of Alizarin red S stain showed that mineralization was significantly increased by treatment for 10 days with 3 mmol/L VPA ( $P = .001$ ) and with 100 nmol/L ( $P = .007$ ) and 200 nmol/L ( $P = .008$ ) TSA (Fig. 3A and B). Mineralization time-course analysis showed a significant reduction in the cell number at 4 days with VPA concentrations of 0.25 mmol/L ( $P = .035$ ), 0.5 mmol/L ( $P = .008$ ), and 1 mmol/L ( $P = .002$ ) and 10 days with 1 mmol/L VPA ( $P = .007$ ) (Fig. 3C). Additionally, a significant reduction in cell numbers was observed at 4, 7, and 10 days after 3 mmol/L VPA administration ( $P < .004$ ). A significant reduction in cell numbers at 4, 7, and 10 days for all concentrations above 12.5 nmol/L TSA ( $P < .035$ ) with a significant reduction at 10 days for 12.5 nmol/L TSA ( $P = .008$ ) was evident when compared with untreated cells (Fig. 3D). No significant difference in cell number for VPA or TSA at 1 day ( $P > .095$ ) was identified for all concentrations investigated (Fig. 3C and D).

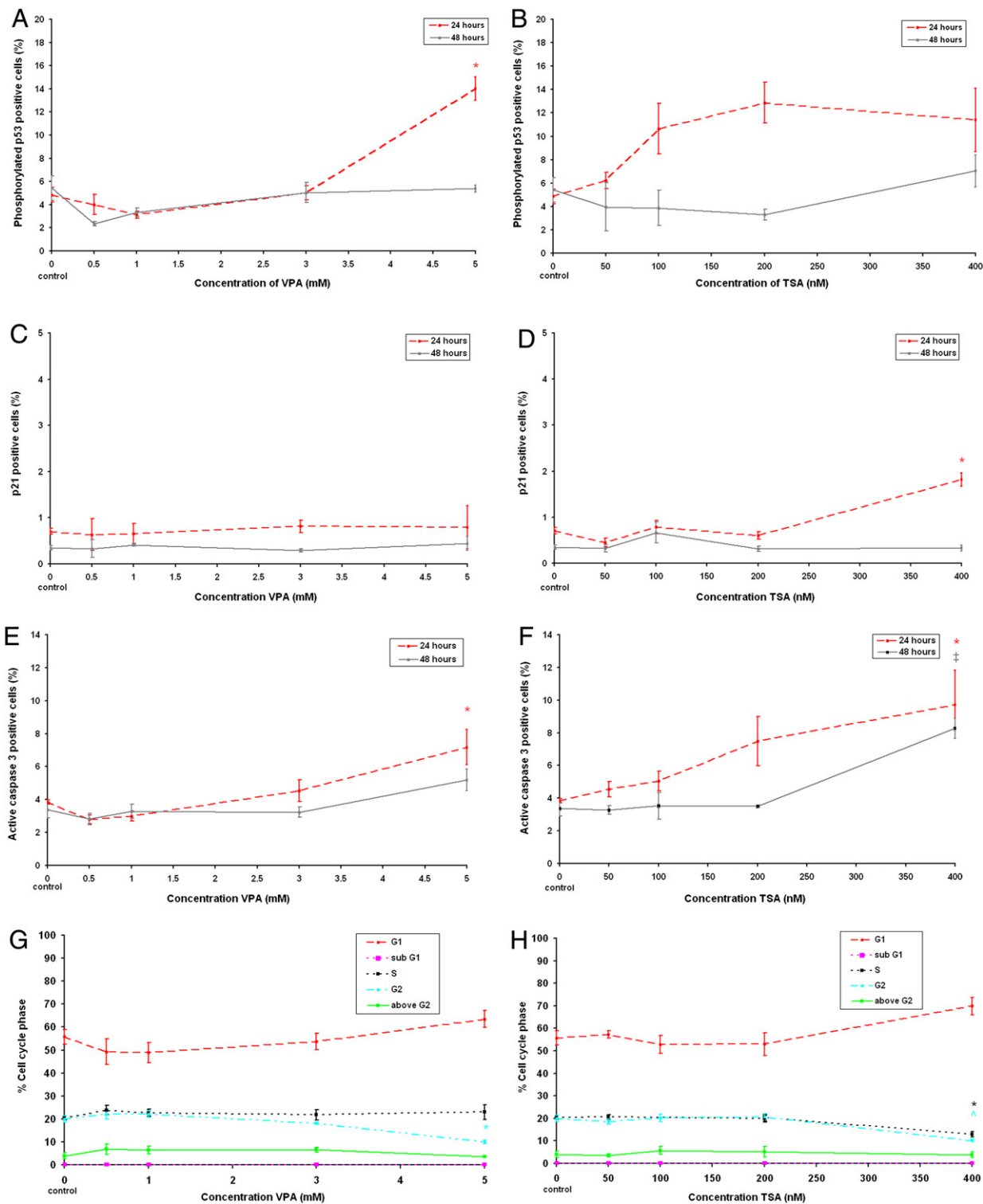
At 10 days, a significant increase in mineralization per cell was evident for all HDACi concentrations with the exception of 0.125 mmol/L VPA ( $P = .968$ ) and 12.5 nmol/L TSA ( $P = .346$ ) when compared with the untreated cells (Fig. 3E and F). VPA and TSA significantly increased mineralization per cell ( $P < .022$ ) in a dose-dependent manner manifest as positive linear regression slopes ( $R^2$  values  $> .86$ ) (Fig. 3E and F).

## Gene Expression

The relative expression of DMP-1 was significantly increased in VPA- ( $P < .03$ ) and TSA- ( $P < .01$ ) treated cells compared with the positive and negative control cultures (Fig. 4A and B). BMP-4 levels were



**Figure 1.** Proliferation and viability of pulp-derived cells cultured in the presence or absence of (A) VPA and (B) TSA. Data represented as mean  $\pm$  standard error measurement (SEM). Asterisks and  $>$  denote a statistically significant difference ( $P < .05$ ) between the experimental groups (VPA and TSA) and the untreated control. The experiment was performed in duplicate on 3 separate occasions.



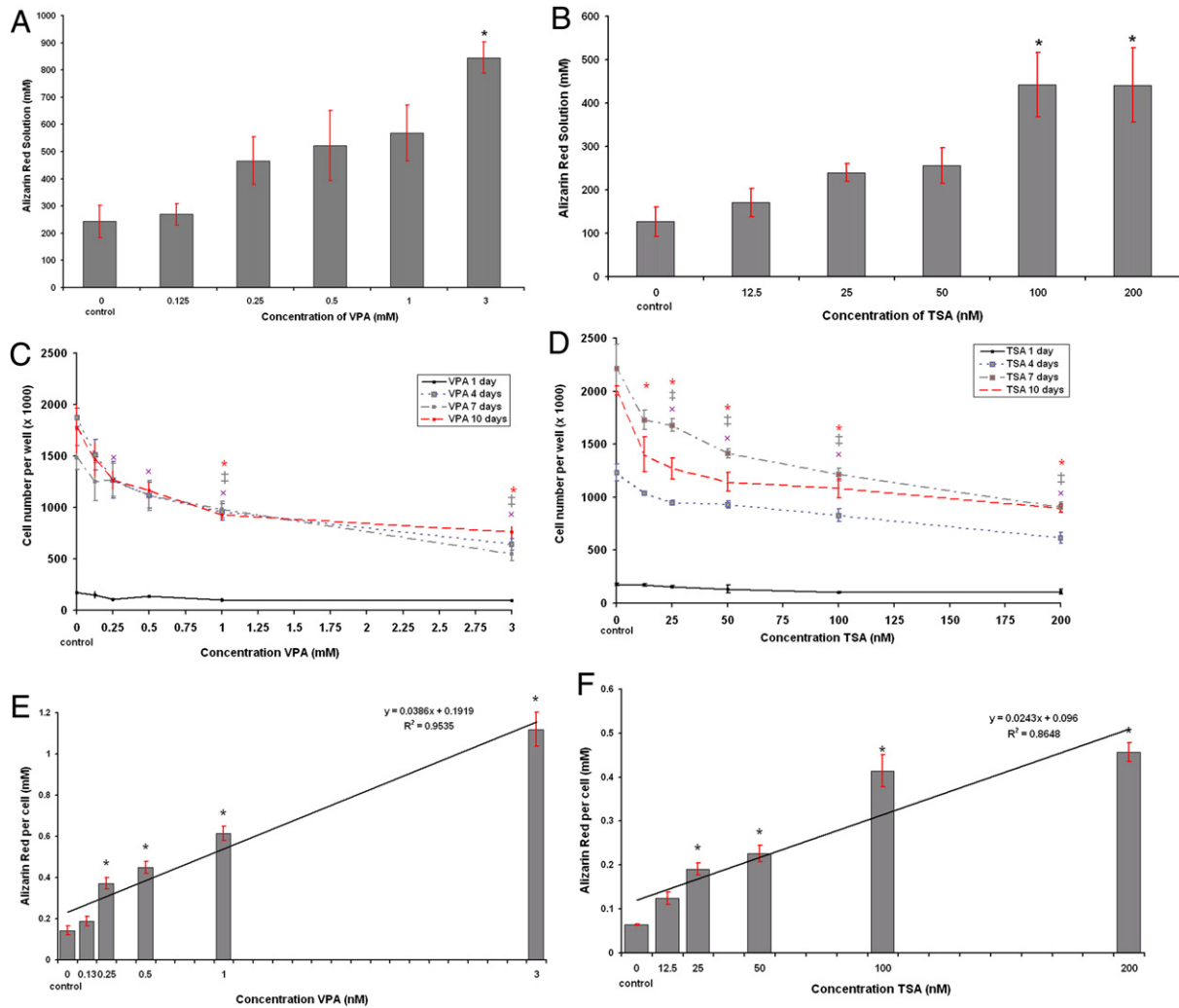
**Figure 2.** The expression of proliferation/apoptosis protein markers and cell cycle analysis in pulp-derived cells cultured in the presence or absence of (A, C, E, and G) VPA and (B, D, F, and H) TSA. All data represented as mean ± SEM. \*A statistically significant difference ( $P < .05$ ) between the experimental groups (VPA and TSA) and the untreated control. Experiments were performed in triplicate on 3 separate occasions.

significantly increased in the VPA-treated cells compared with the negative control ( $P = .027$ ) but not the positive control cultures ( $P = .09$ ) (Fig. 4C). The relative expression levels of TGF- $\beta$ 1 (Fig. 4D) was significantly increased in the positive control ( $P = .017$ ) and VPA-treated ( $P = .026$ ) cells compared with the negative control only.

### Discussion

The present study indicates a novel epigenetic role for HDACis in accelerating regenerative pulp mineralization without affecting cell viability or inducing cell death. HDACis significantly reduced cell proliferation at low concentrations (0.5 mmol/L VPA and 25 nmol/L TSA),





**Figure 3.** Mineralization per cell analysis in pulp-derived cells cultured in the presence and absence of VPA/TSA-supplemented osteogenic medium. (A) VPA and (B) TSA mineralization assay data represented as mean  $\pm$  SEM and statistically significant differences ( $P < .05$ ) between the experimental and control plate are marked (\*). (C) VPA and (D) TSA time-course study data.  $\times$  Statistical significance ( $P < .05$ ) at 4 days.  $\ddagger$  Statistical significance at 7 days. \*Statistical significance at 10 days. (E) VPA and (F) TSA mineral production per cell data represented as mean  $\pm$  SEM. \*Statistically significant differences ( $P < .05$ ) between the experimental and control plate. All experiments were performed in duplicate on 3 separate occasions.

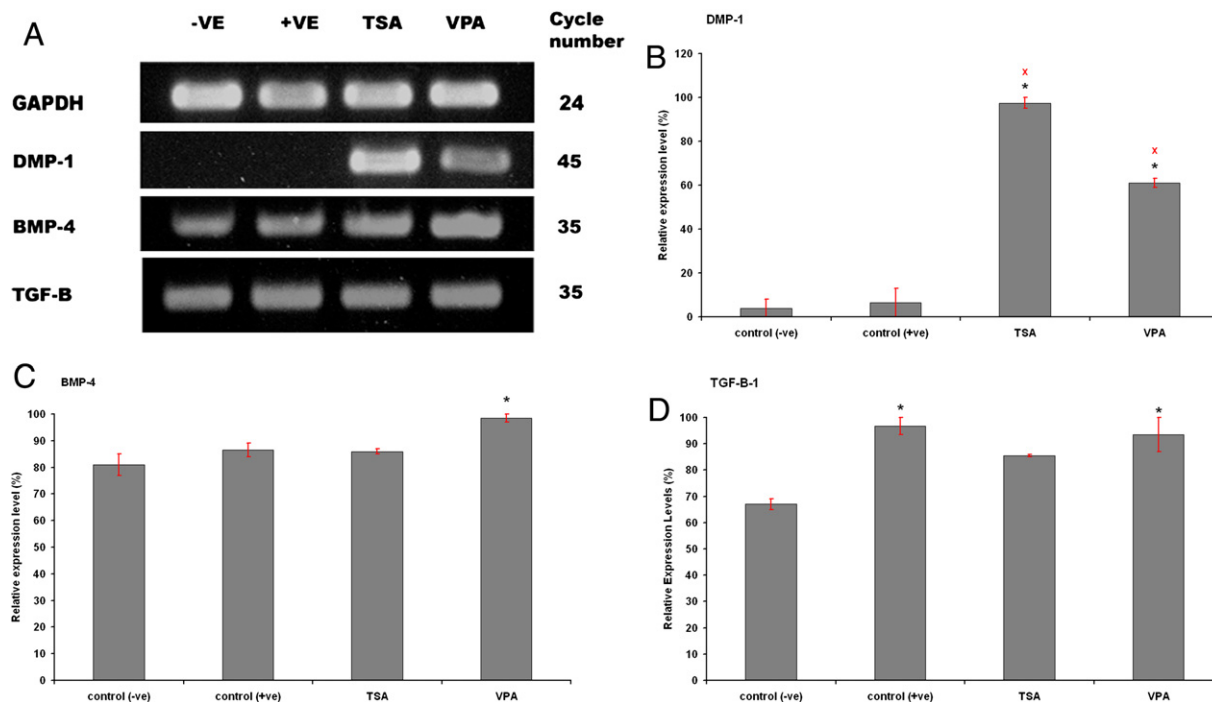
with greater than 85% cell survival showing that pulp cells are sensitive to HDACi-mediated growth inhibition. A progressive significant reduction in cell proliferation was evident with an increasing HDACi concentration to concentrations of 5 mmol/L VPA and 400 nmol/L TSA, indicating a biphasic mode of action and cellular sensitivity. Previously, normal (16) and transformed cells (30) have shown similar HDACi-mediated reduction in proliferation, with HDACi concentration effects varying depending on cell type and the HDACi applied (21, 31).

HDACi-induced growth inhibition, principally of transformed cells, has been reported to be commonly mediated via induction of the cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup> (30, 32), either via p53-dependent or independent pathways (33). We observed a significant increase in p21 levels only at the highest concentration of TSA (400 nmol/L), which was not evident with VPA, which is suggestive of different molecular kinetics or pathways for each HDACi within this pulp-derived cell population. Alternatively, because the HCA technique only captures a “snapshot” of cell activity (24 and 48 hours), these data may indicate that p21 levels are not chronically increased. Notably, the transcription factor p53 was not increased with VPA or TSA, suggesting that a p53-independent mechanism of growth inhibition is operative in

this cell line (33). Interestingly, however, a significant increase in phosphorylated p53 was evident for 5 mmol/L VPA, indicating that a p53-dependent mechanism may still function in orchestrating pulpal cell growth inhibition.

In the current study, despite a progressive reduction in proliferation, VPA and TSA had no significant impact on cell viability (except at 400 nmol/L TSA), showing that HDACi did not induce pulp cell death. Similarly, osteoblast viability was also not reportedly affected by VPA or TSA over a wide range of concentrations (20, 21). The significant increase in active caspase-3 expression at the highest HDACi concentration (5 mmol/L VPA and 400 nmol/L TSA, respectively) indicated HDACi-induced apoptosis, which corroborates the reported reduction in growth and viability at the maximum concentration (34).

Further evidence that VPA and TSA were not inducing cell cycle arrest in these cells was provided by nuclear Hoechst staining and HCA, which showed an alteration in the G1/G2 phase although only at the highest HDACi concentration. However, even at the maximum concentration applied, there was no evidence of cell cycle arrest. HDACi-induced cell cycle arrest has previously been shown in neoplasia studies and at relatively high HDACi concentrations capable of inducing



**Figure 4.** Semiquantitative reverse-transcriptase polymerase chain reaction analysis showing differential gene expression in pulp-derived cells in the presence or absence of VPA and TSA. (A) A gel image showing the expression profile of the housekeeping gene GAPDH and the mineralization/dentinogenic-associated markers DMP-1, BMP-4, and TGF-β-1. The designated cell cycle numbers for the analyses are shown. Graphs showing the relative expression of (B) DMP-1, (C) BMP-4, and (D) TGF-β-1 where the data are represented as mean ± SEM. \*Statistically significant differences ( $P < .05$ ) between the experimental and negative control cultures. × Statistically significant differences between the experimental and positive control cultures at  $P < .05$ . The results represent duplicate experiments performed on 2 separate occasions.

apoptosis (32); however, this arrest is generally not evident in primary cells and at lower HDACi concentrations used for anti-inflammatory and mineralization-inductive effects (17, 21, 35).

Because we observed an HDACi-mediated reduction in cell proliferation without an equivalent increase in apoptotic cell death or cell cycle arrest, we hypothesized that the HDACis were inducing an increase in cell differentiation. Indeed, cellular differentiation was accelerated by VPA and TSA in these pulp-derived cells as shown by Alizarin red S stain quantification, suggesting that as the HDACi reduced growth, mineralized cell (odontoblast-like) differentiation increased dose dependently. Notably, the acceleration of mineral production in these cells occurred with a range of HDACi concentrations below those that significantly reduced viability and induced cell cycle change. Further evidence of cell differentiation was supported by the significantly increased relative expression of the odontoblast-related marker DMP-1 and the general mineralization marker BMP-4 in HDACi-treated cells. The observed increase in DMP-1 suggests that the suppression of HDAC activity during odontoblast-like differentiation can accelerate mineralization. Indeed, HDACis are known to promote differentiation in pluripotent stem cell populations (18), neural progenitor cells (31), and primary osteoblasts (20, 21). Specifically, HDACis stimulate the expression of osteoblast marker genes in primary and osteoblast cell lines (21); however, this has not previously been shown in pulp-derived cells. The authors do accept that previous *in vitro* studies investigating osteoblastic cultures (20, 21) and indeed the currently reported experiments on pulp-derived cells are limited in their clinical relevance by the nature of the 2-dimensional monolayer compared with 3-dimensional tissues. However, the current results provide an exciting framework for future 3-dimensional experimentation. Although the 2 HDACis tested have different chemical structures, specificities and potencies, they both exert similar influences on differentiation, suggest-

ing that they affect similar HDAC enzymes and the expression of a closely related array of genes.

The effective delivery of HDACi drugs to the damaged pulp will require a suitable carrier material. Logically, HDACis could be integrated into existing dental restorative materials similar to the way in which other agents have been incorporated with and without the assistance of the material manufacturer (2, 26, 36–38). This approach may be useful for developing a new regenerative restorative material that can be used in direct contact vital pulp treatment and warrants further *in vitro* studies investigating the physical properties of the integrated material and the stability of HDACi release.

In this study, we showed that 2 HDACis reduce proliferation and increase mineralization in a dose-dependent manner in pulp-derived cells while not significantly influencing cell viability and cell cycle. Notably, the combined increase in mineral per cell and dentinogenic gene expression indicates that these inhibitors can epigenetically modulate the cell encouraging acceleration and odontoblast-like differentiation. Because HDACis are currently being used as novel treatment approaches in several clinical areas, they represent interesting therapeutic candidates for regenerative biomaterials within restorative dentistry.

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The authors deny any conflicts of interest related to this study.

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## CHAPTER 5

### PUBLICATION 4: ORIGINAL SCIENTIFIC MANUSCRIPT

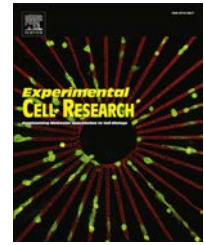
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The literature highlighted differences between transformed and primary cell responses to HDACis (Chapter 2). An HDACi-induced dose-dependent promotion of mineralisation in pulp-derived cells was demonstrated (Chapter 4) with a significant reductions in cell growth, cell viability and cell cycle alteration evident at the higher HDACi concentrations. As a result, it was hypothesised that HDACis would induce reparative responses in primary DPCs at concentration and dose levels which did not stimulate significant cytotoxic effects. The subsequent aim was to replicate and expand the MDPC-23 experimentation to primary DPCs by quantitatively analysing mineralisation responses by focusing on a range of assays investigating cell viability, cell growth, cell apoptosis and mineralisation-associated gene/protein marker expression.

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## Research Article

# Histone deacetylase inhibitors epigenetically promote reparative events in primary dental pulp cells

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## ABSTRACT

Application of histone deacetylase inhibitors (HDACi) to cells epigenetically alters their chromatin structure and induces transcriptional and cellular reparative events. This study investigated the application of two HDACi, valproic acid (VPA) and trichostatin A (TSA) on the induction of repair-associated responses in primary dental pulp cell (DPC) cultures. Flow cytometry demonstrated that TSA (100 nM, 400 nM) significantly increased cell viability. Neither HDACi was cytotoxic, although cell growth analysis revealed significant anti-proliferative effects at higher concentrations for VPA (>0.5 mM) and TSA (>50 nM). While high-content-analysis demonstrated that HDACi did not significantly induce caspase-3 or p21 activity, p53-expression was increased by VPA (3 mM, 5 mM) at 48 h. HDACi-exposure induced mineralization per cell dose-dependently to a plateau level (VPA-0.125 mM and TSA-25 nM) with accompanying increases in mineralization/dentinogenic-associated gene expression at 5 days (DMP-1, BMP-2/-4, Nestin) and 10 days (DSPP, BMP-2/-4). Both HDACis, at a range of concentrations, significantly stimulated osteopontin and BMP-2 protein expression at 10 and 14 days further supporting the ability of HDACi to promote differentiation. HDACi exert different effects on primary compared with transformed DPCs and promote mineralization and differentiation events without cytotoxic effects. These novel data now highlight the potential in restorative dentistry for applying low concentrations of HDACi in vital pulp treatment.

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## Introduction

Histone deacetylase inhibitors (HDACi) regulate the acetylation of DNA-associated proteins and subsequently alter chromatin architecture, resulting in changes to transcriptional activity [1–3]. Epigenetically-induced HDACi-modifications have beneficial effects on tissue repair mechanisms including the promotion of stem cell recruitment and the induction of differentiation and mineralization processes [4–6], as well as modulating inflammation [7]. Therapeutically, these properties of HDACi could be harnessed to augment

biologically-driven dental restorative approaches [8,9] with potential to improve the predictability of healing outcomes. Regenerative techniques which preserve pulp vitality have recently been emphasized [10], however, currently they have poor prognosis [11] potentially in part, due to the failure of materials to control inflammation [12] and enable consistent new matrix deposition [13,14].

Recent studies [15,16] demonstrate that HDACi promote mineralization in dental pulp cell lines, suggesting their therapeutic potential. However, variances were documented in cell resistance [17,18], cell toxicity [17,18] and inhibitor concentration [17,18]

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between HDACi-induced responses in primary and transformed cells. This altered cellular sensitivity has been the basis of multiple studies investigating HDACi as potential anti-cancer agents [1,3,5], acting by the promotion of cell death pathways in transformed cell lines [19,20], but not in primary cells [17,18,21]. Because of these contrasting cellular responses, it is essential that the effects of HDACi are now investigated in primary dental pulp cells (DPCs), which provide greater translational and clinical relevance.

A host of HDACi has been identified with a subgroup, of mainly pan-inhibitors, now engaged in clinical trials [1]. Although, valproic acid (VPA) and trichostatin A (TSA) are considered pan-inhibitors they have different chemical structures, specificities and potencies. However, both have been reported to exert similar influences on differentiation suggesting that they affect similar HDAC enzymes and the expression of a closely related array of genes [4]. Chemically, the structure of TSA is based around a hydroxamic acid structure and it is regarded as a general class I and II HDAC inhibitor, while VPA possesses a carboxylic acid functional group and does not inhibit all class II HDAC enzymes (HDAC6 or HDAC10) [22]. Reported differences in cell behavior in response to different HDACi are likely to be related to the specificity of each inhibitor [1,4,22].

Based on previous studies, we hypothesize that the two HDACi investigated, VPA and TSA, induce reparative responses in primary DPCs at concentration levels which do not stimulate significant anti-proliferative, apoptotic or necrotic effects.

## Material and methods

### Primary cell isolation and culture

The primary dental pulp cells (DPCs) used in this study were isolated from the pulpal tissue of freshly extracted rodent incisor teeth using enzymatic disaggregation [23]. All teeth were dissected from male Wistar Hannover rats aged 25–30 days and weighing 120–140 g. Post-extraction, the pulp tissue was extirpated from the teeth and minced aseptically into pieces of approximately 1.0 mm<sup>2</sup>. The tissue was transferred into a 50 ml Falcon™ tube (BD Biosciences, Oxford, UK) containing 4 ml Hank's balanced salt solution (Sigma-Aldrich, Arklow, Ireland) (2.5 g/l trypsin, 0.2 g/l ethylenediaminetetraacetic acid (EDTA) and 4Na) prior to incubation at 37 °C at 95% relative humidity in a 5% CO<sub>2</sub> atmosphere for 30 min (MCO-18AC incubator, Sanyo Electric, Osaka, Japan). In order to improve dissociation, the cells were constantly agitated during digestion (MACSmix tube rotator, Miltenyi Biotec, Surrey, UK) prior to an equal volume of supplemented  $\alpha$ -MEM (Biosera, East Sussex, UK) containing 1% penicillin/streptomycin (Sigma-Aldrich) and 10% (v/v) foetal calf serum (FCS) (Biosera) being added. In order to obtain a single cell suspension, the digest was passed through a 70  $\mu$ m cell sieve (BD Biosciences) and the cells pelleted by centrifugation at 1200 rpm for 3 min, prior to re-suspension in 1 ml supplemented  $\alpha$ -MEM. Subsequently, the DPCs were expanded in culture to passage 2 for use in all subsequent experiments. Unless otherwise stated, all cultures were maintained in supplemented  $\alpha$ -MEM at 95% relative humidity in a 5% CO<sub>2</sub> atmosphere at 37 °C.

### HDACi

A 1 M solution of VPA (2-propylpentanoic acid sodium salt) (Sigma-Aldrich) in phosphate buffered saline (PBS) (Sigma-Aldrich) was diluted to a range of experimental concentrations (30  $\mu$ M–5 mM) with supplemented  $\alpha$ -MEM. A 5 mM solution of TSA (7-[4-(Dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamido) (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) was diluted in PBS prior to dilution to experimental concentrations (3 nM–400 nM) in supplemented  $\alpha$ -MEM.

### Evaluation of cell growth and viability

DPCs were seeded at a density of  $1 \times 10^5$  in 6-well plates (Sarstedt, Wexford, Ireland) for 24 h to allow attachment and initial proliferation. At 24 h (experimental day 0), the cells were cultured in supplemented  $\alpha$ -MEM for a further 1, 3 and 6 days. VPA (0.25, 0.5, 1, 3 and 5 mM) and TSA (25, 50, 100, 200 and 400 nM) were added to the supplemented  $\alpha$ -MEM, at day 0, for the initial 24 h. The 1 day experimental cultures were harvested after 24 h, while in the 3 and 6 day experiments the HDACi-supplemented medium was replaced after 24 h with supplemented  $\alpha$ -MEM for a further 2 and 5 days. The control samples contained cells cultured in supplemented  $\alpha$ -MEM without HDACi. Cultures were detached with trypsin/EDTA (Sigma-Aldrich) and stained with 0.4% trypan blue (Sigma-Aldrich) prior to cell counting using a standard hemocytometer (Bright-Line™ Neubauer Haemocytometer, Hausser Scientific, PA, USA). Three independent experiments ( $n=3$ ) were performed in triplicate for each HDACi concentration and time interval respectively, and DPC number and viability recorded.

### Flow cytometry (FC)

FC detection (BD FACSCanto II, BD Biosciences) of annexin V (AV) binding and propidium iodide (PI) staining (Annexin V-FITC Kit, BD Bioscience) was performed to assess viability, necrosis and secondary apoptosis. Cells were seeded at a density of  $1 \times 10^5$  on 6-well plates and cultured in supplemented  $\alpha$ -MEM for 24 h, prior to addition of VPA (1 and 5 mM) or TSA (100 and 400 nM) for a further 24 h. The experimental time-point was chosen as significant early viability changes have been previously reported with HDACi [4] and apoptosis at 24 h. Untreated cells in supplemented  $\alpha$ -MEM served as a negative control. Cells in supplemented  $\alpha$ -MEM with 6  $\mu$ M camptothecin (Sigma-Aldrich) were employed as a positive control to confirm experimental apoptosis. Cells were detached with trypsin/EDTA, washed twice with PBS and suspended in a  $1 \times$  AV binding buffer solution (BD Bioscience) at a concentration of  $1 \times 10^6$  cells/ml. A 100  $\mu$ l aliquot of the cell suspension ( $1 \times 10^5$ ) was transferred to a 5 ml polystyrene tube (BD Bioscience), prior to incubation at room temperature with 5  $\mu$ l FITC-AV and 5  $\mu$ l PI for 15 min. Thereafter, 400  $\mu$ l of  $1 \times$  AV binding buffer solution was added to the tube and the cells analyzed by FC within 1 h of harvesting. Excitation was performed at 488 nm and the emission filters used for AV-FITC and PI were 530/530 and 585/545, respectively. The data were analyzed using FC software (FloJo, Tree Star, OR, USA) and the PI staining intensity plotted against FITC intensity. Three independent experiments ( $n=3$ ) were performed in triplicate

with 10,000 cell events being measured for each experimental/control group.

### High content analysis (HCA)

To determine the influence of VPA and TSA concentration on the DPC cycle, proliferation and apoptotic marker expression at 24 and 48 h, HCA was performed. These early time-points were based on a previous study [9]. The cells were seeded at a density of  $4 \times 10^3$  in 96-well plates (Sigma-Aldrich) for 24 h, cultured with supplemented  $\alpha$ -MEM containing VPA (0.25, 0.5, 1, 3 and 5 mM) and TSA (25, 50, 100, 200 and 400 nM). Negative control samples contained cells cultured in supplemented  $\alpha$ -MEM without HDACi. Positive control cultures had supplemented  $\alpha$ -MEM with HDACi, but no cells were seeded in those wells. Cells were treated with VPA/TSA for 24 h prior to either fixation (24 h experimental samples) or further incubation for 24 h (48 h experimental samples) in supplemented  $\alpha$ -MEM prior to fixation. At the designated end-point, the medium was removed and cells fixed in 1% formaldehyde (Sigma-Aldrich) in PBS solution for 30 min. Subsequently, the cells were processed (Imagen Biotech, Manchester, UK) by permeabilisation and stained with antibodies against active caspase-3 (Cat# ab13847; Abcam, Cambridge, UK), p53 (Cat# PN8400602; Thermo Fisher Scientific, Leicestershire, UK) and p21 (Cat# PN8400602; Thermo Fisher Scientific). All antibodies were diluted to 500  $\mu$ g/ml digitonin/PBS solutions. Cross reactions were visualized using two fluorophore conjugated secondary antibodies, namely goat anti-mouse 488 (Invitrogen, Paisley, UK) and goat anti-rabbit 594 (Invitrogen) either in isolation or in combination. For cell cycle analysis, DNA was stained with Hoechst 33342 at 2.5  $\mu$ g/ml (Invitrogen). The 96-well plates were analyzed on an Arrayscan (Cellomics, Thermo Fisher Scientific) using a compartmental analysis algorithm. Three independent experiments ( $n=3$ ) were performed in triplicate for each antibody investigated.

### Mineralization assay

Cells were seeded at a density of  $6 \times 10^4$  in 6-well plates and cultured in supplemented  $\alpha$ -MEM for 72 h until 60–70% confluent. At 72 h, the DPCs were cultured (day 0) in a mineralization medium (supplemented  $\alpha$ -MEM including 50  $\mu$ g/ml ascorbic acid, 0.1  $\mu$ M dexamethasone and 10 mM  $\beta$ -glycerophosphate) with the addition of VPA (30, 60, 125, 250 and 500  $\mu$ M) or TSA (3, 6, 12.5, 25 and 50 nM) for the initial 24 h of culture. The HDACi-supplemented mineralization medium was removed after 24 h prior to culture with an HDACi-free mineralization medium for a further 13 days. This time-point was selected as rat DPCs in mineralizing culture require 14 days to secrete quantitative mineral that can be discriminatively measured by Alizarin red staining. Control samples contained cells cultured in mineralization medium without HDACi. The mineralization medium was changed every 3 days. For analysis, cultures were washed 3 times for 5 minutes in PBS and fixed in 10% formaldehyde for 15 min, washed with distilled water and finally stained with 1.37% (w/v) alizarin red S (Millipore, Cork, Ireland) (pH 4.2) for 15 min at room temperature. Excess stain was removed by washing with distilled water; the residual stain was solubilised in 10% acetic acid (Millipore) and the supernatant transferred to a transparent 96-well plate (Corning, Amsterdam, The Netherlands). The

intensity of stain was quantified spectrophotometrically at 405 nm [24] (Tecan Genios Spectrophotometer, Unitech, Dublin, Ireland).

Mineralization was normalized by cell number. In a parallel study, DPCs were cultured using an identical protocol to the aforementioned mineralization assay for 14 days. After 14 days, the cells were not fixed, but detached with trypsin/EDTA and stained with 0.4% trypan blue, prior to cell counting using a standard hemocytometer. Mineral production per cell data for all concentrations of VPA and TSA were subsequently calculated based on quantification of alizarin red S stain extracted from each experimental well and corresponding cell counts. Three independent experiments ( $n=3$ ) were performed in triplicate for both the mineralization assay and cell counts, respectively.

### RNA isolation and cDNA synthesis

Cells were seeded at a density of  $6 \times 10^4$  onto a 6-well culture dish and cultured in 2 ml of supplemented  $\alpha$ -MEM for 72 h until 60–70% confluent. At 72 h (experimental day 0), the cells were cultured for 24 h in supplemented mineralization medium containing 125  $\mu$ M VPA or 25 nM TSA, before incubation with an HDACi-free mineralizing medium for a further 4 or 9 days (experimental day 5 and 10). The 2 time-points selected for gene expression analyses were planned to precede the later protein expression experimentation. Control samples contained cells cultured in mineralization medium without HDACi. Cultures were detached with trypsin/EDTA, homogenized for 30 s using a T10 basic S2-Ultra-Turrax tissue disrupter (IKA, Staufen, Germany) and RNA extracted using the RNeasy mini kit (Qiagen, West Sussex, UK). RNA was isolated on the RNeasy mini-column assembly (Qiagen), prior to conversion to single-stranded cDNA using the Omniscript kit (Qiagen) and oligo-dT primer (Ambion, Applied Biosciences, Dublin, Ireland). Synthesized cDNA was concentrated and purified by centrifugation with Microcon YM-30 filters (Millipore) and cDNA concentrations determined spectrophotometrically at 260 nm (Nanodrop 2000, Thermo Fisher Scientific).

### Semi-quantitative reverse transcriptase-polymerase reaction (sq-RT-PCR)

Sq-RT-PCR analysis was performed for rat genes using specific primers (Invitrogen). The primer sequences and the product sizes are listed in Table 1 for each experimental target gene; dentine matrix phosphoprotein-1 (DMP-1), dentine sialophosphoprotein (DSPP), bone morphogenic protein-2 (BMP-2), bone morphogenic protein-4 (BMP-4), nestin and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each PCR included; 12.5  $\mu$ l REDTaq ReadyMix™ PCR Reaction Mix (with MgCl<sub>2</sub>) (Sigma-Aldrich, Arklow, Ireland), 0.5  $\mu$ l Forward/Reverse primer, 10  $\mu$ l molecular grade water and 50 ng of template cDNA. PCRs were performed using the GS-1G-Storm thermal cycler (G-Storm, Surrey, UK) and subjected to a designated number (21–37) of amplification cycles, where a typical cycle was 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. Following thermal-cycling, amplified products were electrophoresed on a 1.5% agarose gel containing gel red (Biotium, CA, USA), before visualization under ultra-violet light with the Alphamager-Mini system™ (Alphainnotech, Cell Biosciences, CA, USA). Captured gel images were imported into



**Table 1 – Primer sequences used for primary rat DPCs gene expression analyses.**

Gene	Primer sequence (5'→3')	Size bps	Accession number
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	(F)-CGATCCCGTAACATCAAAT (R)-GGATGCAGGGATGATGTTCT	391	NM_017008
Dentine matrix phosphoprotein-1 (DMP-1)	(F)-TGCATTTTGAAGTGCTCGC (R)-CCTCCTGCTTGGTGTGGTT	248	NM_203493.3
Dentine sialophosphoprotein (DSPP)	(F)-TGCATTAAGTGCTCGC (R)-CCTCCTGCTTGGTGTGGTT	277	NM_012790.2
Bone morphogenic protein-2 (BMP-2)	(F)-TGAACACAGCTGGTCTCAGG (R)-TGAACACAGCTGGTCTCAGG	320	NM_012987.1
Bone morphogenic protein-4 (BMP-4)	(F)-ATGTGACACGGTGGAAACT (R)-AGTCCACGTAGAGCGAATGG	323	NM_012827.2
Nestin	(F)-CATTAGATGCTCCCGAGA (R)-AATCCCATCTACCCCACT	285	NM_012987.1

AlphaView™ image analysis software (Alphainnotech) for densitometric analysis. Volume densities of amplified products were normalized to GAPDH expression. Two independent experiments ( $n=2$ ) were performed in replicate for both time intervals.

### Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded at a density of  $6 \times 10^4$  in 6-well plates and cultured in supplemented  $\alpha$ -MEM for 72 h until 60–70% confluent. At 72 h, the DPCs were cultured (day 0) in a mineralization medium with the addition of VPA (60, 125, 250 and 500  $\mu$ M) or TSA (6, 12.5, 25 and 50 nM) for the initial 24 h of culture. The HDACi-supplemented mineralization medium was removed after 24 h prior to culture with an HDACi-free mineralization medium for further 5, 9 or 13 days. The quantification of mineralization associated proteins was investigated at 2 time-intervals prior to the 14 day mineralization end point, to establish a time-course analysis of OP and BMP-2 expression. Control samples contained cells cultured in mineralization medium without HDACi. The mineralization medium was changed every 3 days. The OP and BMP-2 levels in the culture medium at experimental day 6, 10 and 14 were analyzed with a commercial quantitative sandwich ELISA technique (R&D Systems, Abingdon, UK) according to the manufacturer's instructions and absorbance measured at 450 nm. The protein concentration was normalized by cell number at each time interval. At the end of the experimental period (6, 10 or 14 days), the supernatant was removed for ELISA and the residual attached DPCs were harvested with trypsin/EDTA and stained with 0.4% trypan blue, prior to cell counting using a standard hemocytometer. Protein production per cell was subsequently calculated based on colorimetric quantification extracted from each experimental well and corresponding cell counts. Three independent experiments ( $n=3$ ) were performed in triplicate for each experimental concentration and time interval.

### Statistical analyses

One-way analysis of variance (ANOVA) and Tukey's post-hoc tests were used for all experiments to determine the influence of HDACi concentration ( $p < 0.05$ ) on the cells using SigmaStat 12.0.1 software (SPSS, IL, USA). Mean and standard deviation were used for statistical analysis. To investigate the dose-dependent effects

of VPA and TSA, linear regression analyses were applied to the mineralization per cell data.

## Results

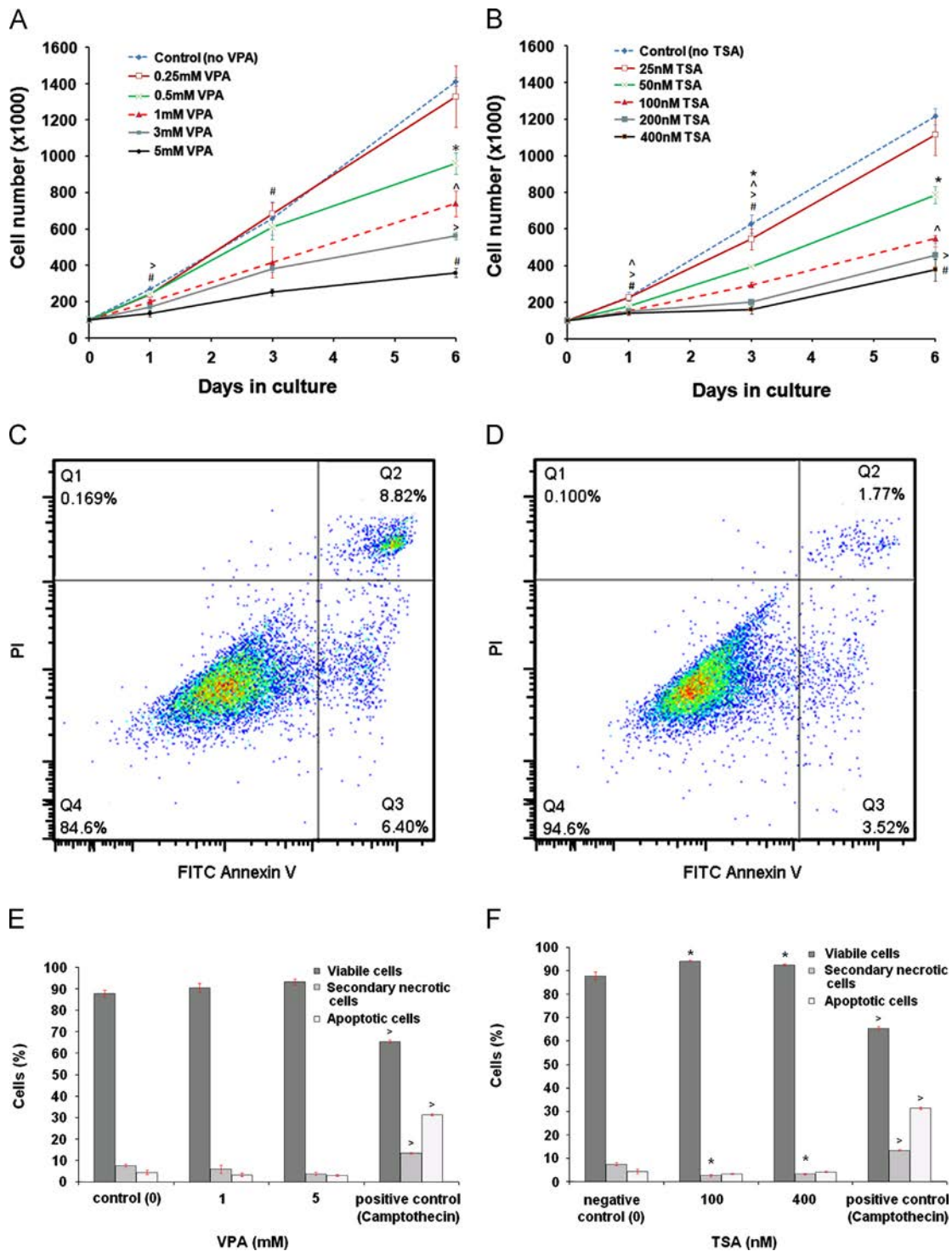
### Cell viability and proliferation in response to HDACi

Cell viability (Trypan blue exclusion) was not significantly reduced at any concentration of VPA ( $p > 0.107$ ) or TSA ( $p > 0.138$ ) applied compared with the control at 1, 3 or 6 days. However, growth was significantly inhibited at 24 h by 3 mM/5 mM VPA ( $p=0.03/p=0.004$ ) and 100, 200, 400 nM TSA ( $p < 0.021$ ), at 3 days by 5 mM VPA ( $p=0.008$ ) and all concentrations of TSA except 25 nM ( $p=0.513$ ). At 6 days, proliferation was significantly reduced with all experimental concentrations except 250  $\mu$ M VPA ( $p=0.974$ ) and 25 nM TSA ( $p=0.869$ ), compared with HDACi-free control cultures (Fig. 1A and B). Notably, while the cell viability data assessed by FC (Fig. 1C–F), was not significantly affected at any VPA concentration ( $p=0.605/p=0.139$ ); viability was significantly increased at 100/400 nM TSA ( $p=0.006/p=0.032$ ) (Fig. 1E and F).

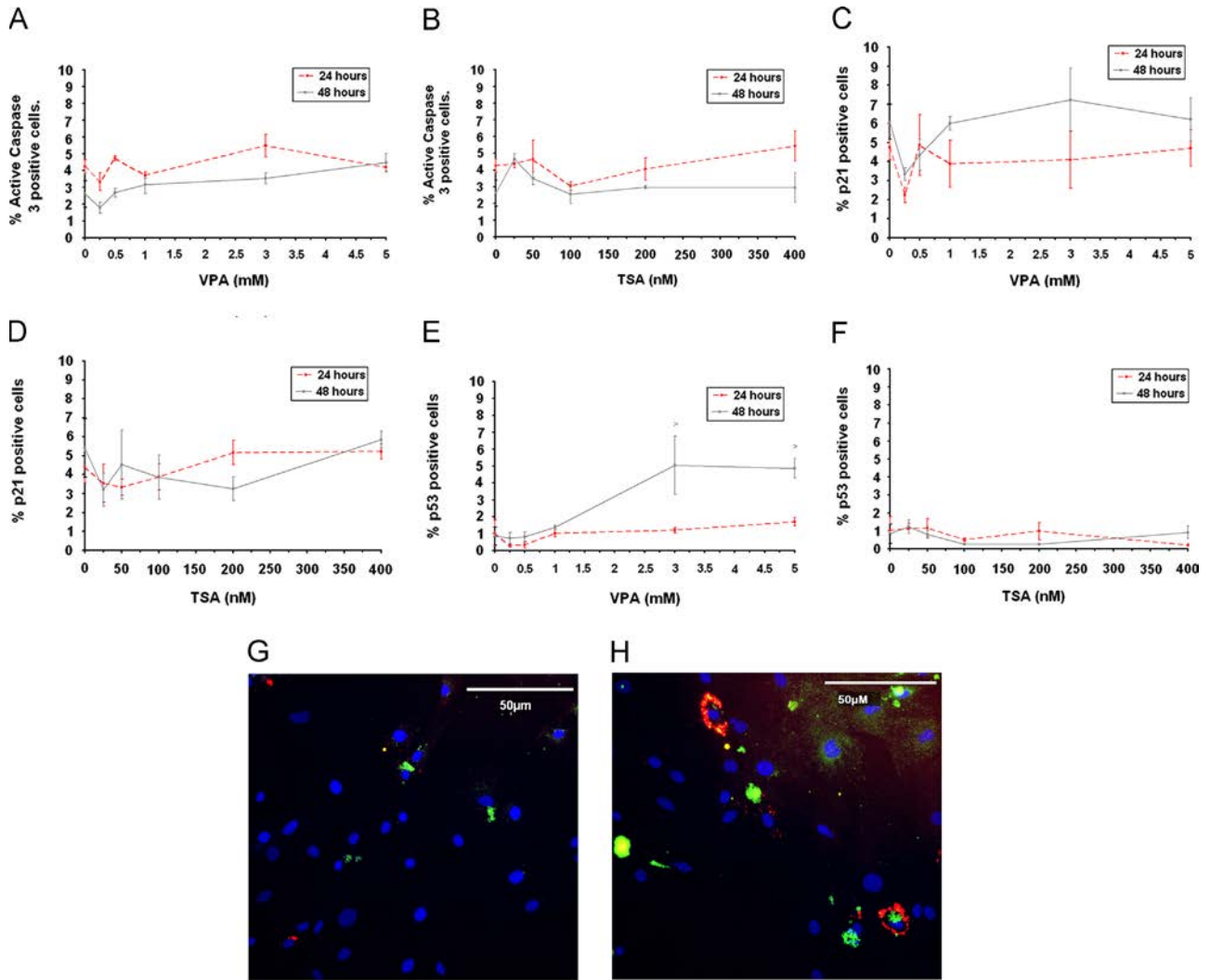
Apoptosis was investigated by both FC and HCA of active caspase-3. FC data demonstrated no significant increase in apoptosis following VPA ( $p=0.741$ ) or TSA ( $p=0.577$ ) exposure at 24 h (Fig. 1E and F). HCA showed no significant increase of caspase-3 at 24 or 48 h for any concentration of VPA ( $p > 0.298$ ) or TSA ( $p > 0.195$ ) (Fig. 2A and B). HDACi-mediated growth inhibition in transformed cells has been attributed to induction of p21 [25] and p53 [26], however, no differences in transcription factor p21 expression were demonstrated for VPA/TSA at 24 h ( $p > 0.766/0.875$ ) or 48 h ( $p > 0.763/0.773$ ) (Fig. 2C and D). There were no significant changes in p53 expression at 24 h with VPA/TSA ( $p > 0.658/p > 0.755$ ) or 48 h with TSA ( $p > 0.406$ ) (Fig. 2E and F), while at 48 h, a significant increase in p53 was identified at 3 mM ( $p=0.026$ ) and 5 mM ( $p=0.035$ ) VPA (Fig. 2E, G and H).

### Cell differentiation

At 14 days, significant increases in mineralization per cell were evident for all VPA ( $p < 0.008$ ) and TSA ( $p < 0.016$ ) supplemented concentrations with the exception of 3 nM TSA ( $p=0.121$ ) and



**Fig. 1 – Growth and viability of primary DPCs cultured with HDACi.** Experimental HDACi, VPA (A) and TSA (B) supplemented cultures for 1, 3 and 6 days at a range of concentrations. Asterisk (\*) for 0.5 mM VPA and 50 nM TSA concentrations, arrow (^) for 1 mM VPA and 100 nM TSA, symbol (>) for 3 mM VPA and 200 nM TSA and symbol (#) for 5 mM VPA and 400 nM TSA represent statistically significant differences ( $p < 0.05$ ) between the experimental groups and the HDACi-free control. FC images for control (no HDACi) (C) and 100 nM TSA (D). Viable cells were observed in the left lower quadrant, apoptotic cells in the right lower quadrant and secondary necrotic cells in the right upper quadrant and charted as % total number of cells. Positive (6  $\mu$ M camptothecin), negative control (0 VPA/TSA) and selected concentrations of VPA (1 mM, 5 mM) (E) and TSA (100 nM, 400 nM) (F) were investigated by FC at 24 h. Asterisk (\*) represents statistically significant differences ( $p < 0.05$ ) between the experimental groups (VPA and TSA) and HDACi-free control. Symbol (>) represents statistically significant differences between all groups and the positive control ( $p < 0.0001$ ). All charted data are represented as mean  $\pm$  SEM.



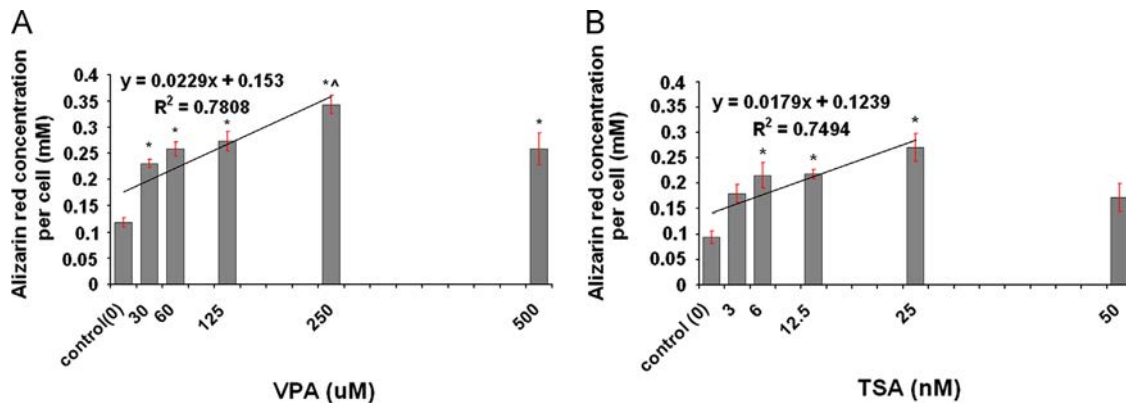
**Fig. 2** – Expression of proliferation and apoptotic protein markers in primary DPCs cultured in the presence of a range of VPA and TSA concentrations. Negative controls contained cell cultures not supplemented with HDACi, positive controls contained no cells only media and HDACi. The number of cells expressing the marker-protein was recorded as a % of the total number of cells scanned by HCA; active caspase-3 expression after addition of VPA (A) and TSA (B) at 24 and 48 h, p21 expression after VPA (C) and TSA (D) at 24 and 48 h and nuclear p53 after VPA (E) and TSA (F) at 24 and 48 h. Symbol (>) represents statistically significant differences ( $p < 0.05$ ) between the experimental groups (VPA and TSA) and the HDACi-free negative control. Representative images for the stained p53 HCA for control (G) and 5 mM VPA at 48 h (H); cell nuclei stained blue, p53 stained green and phosphorylated p53 stained red, which highlights a visible increase in expression of the transcription factor between the control and experimental plates (original magnification  $10\times$ ).

50 nM TSA ( $p=0.180$ ), compared with HDACi-free control cultures (Fig. 3A and B). Additionally, 250  $\mu\text{M}$  VPA supplementation demonstrated a significant increase in mineralization compared with all other VPA concentrations ( $p < 0.048$ ), except 125  $\mu\text{M}$  ( $p=0.124$ ). VPA and TSA significantly increased mineralization per cell ( $p < 0.025$ ) in a dose-dependent manner up to a plateau of 250  $\mu\text{M}$  for VPA and 25 nM for TSA as demonstrated by positive linear regression analyses (Fig. 3A and B).

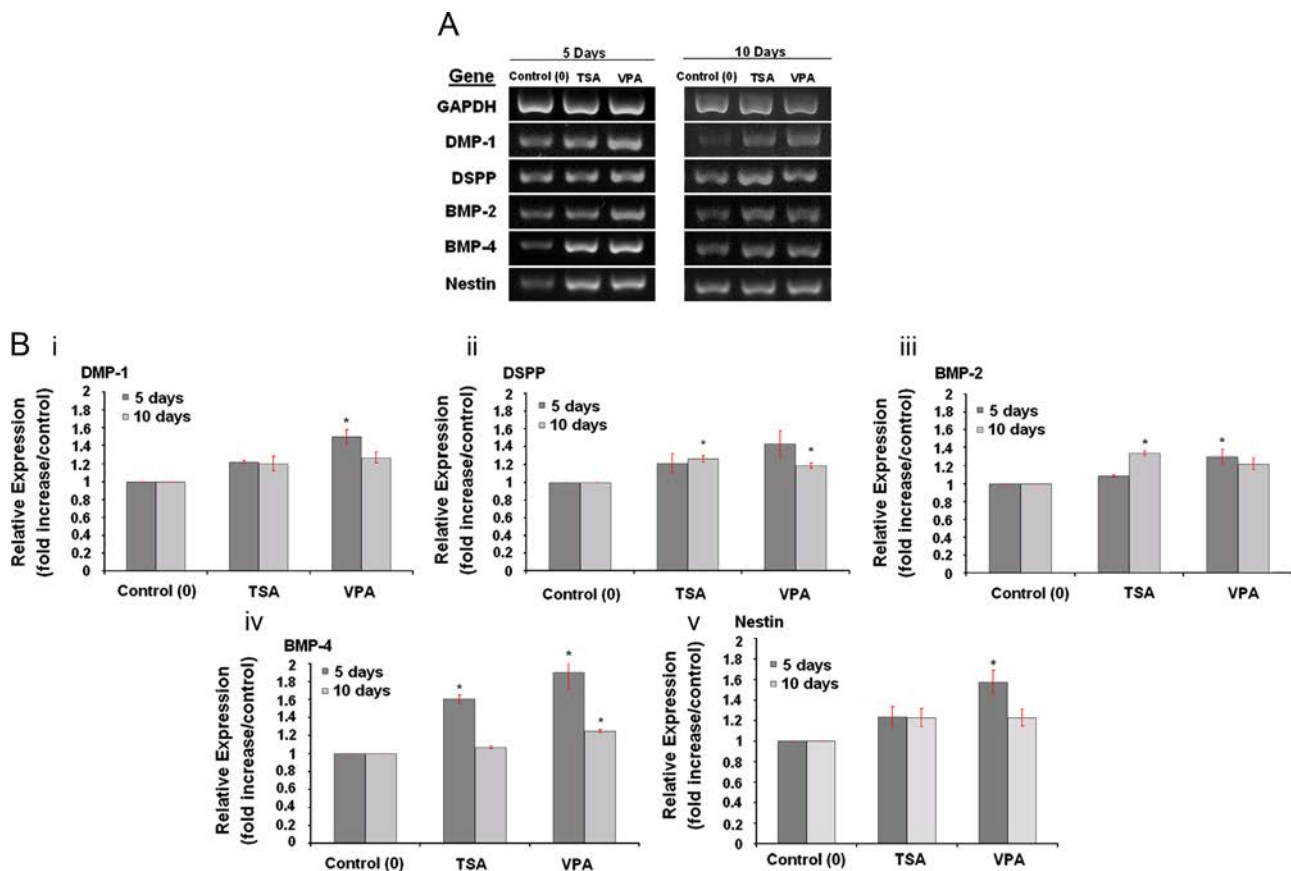
As a dose-dependent HDACi-induced mineralization response in DPCs was observed, further analysis of differentiation was assessed by gene (Fig. 4A) and protein expression. VPA-treated cultures significantly increased their relative expression of DMP-1 ( $p=0.011$ ), BMP-2 ( $p=0.041$ ), BMP-4 ( $p=0.03$ ) and Nestin ( $p=0.037$ ) at 5 days and DSPP ( $p=0.03$ ), BMP-4 ( $p=0.002$ ) at 10 days

compared with HDACi-free equivalent cultures (Fig. 4Bi–v). Significant increases were demonstrated in the relative expressions of BMP-4 ( $p=0.049$ ) at 5 days and DSPP ( $p=0.012$ ), BMP-2 ( $p=0.017$ ) at 10 days with TSA treated cells compared with the control cultures. No differences in the relative expression levels of DMP-1 and Nestin (Fig. 4B) were detected with TSA at 5 or 10 days. Increased OP protein levels by 10 days were evident in response to exposure to 25/50 nM TSA ( $p=0.049/p=0.032$ ) and to all concentrations of VPA ( $p < 0.046$ ) (Fig. 5Ai and Bi). At 10 days, there was also a significant increase in the expression of BMP-2 protein levels for all VPA concentrations ( $p < 0.046$ ), except 60  $\mu\text{M}$  ( $p=0.099$ ) and all TSA concentrations ( $p < 0.039$ ), except 6 nM ( $p=0.061$ ), compared with HDACi-free control cultures (Fig. 5Aii and Bii). At 14 days, BMP-2 protein levels were

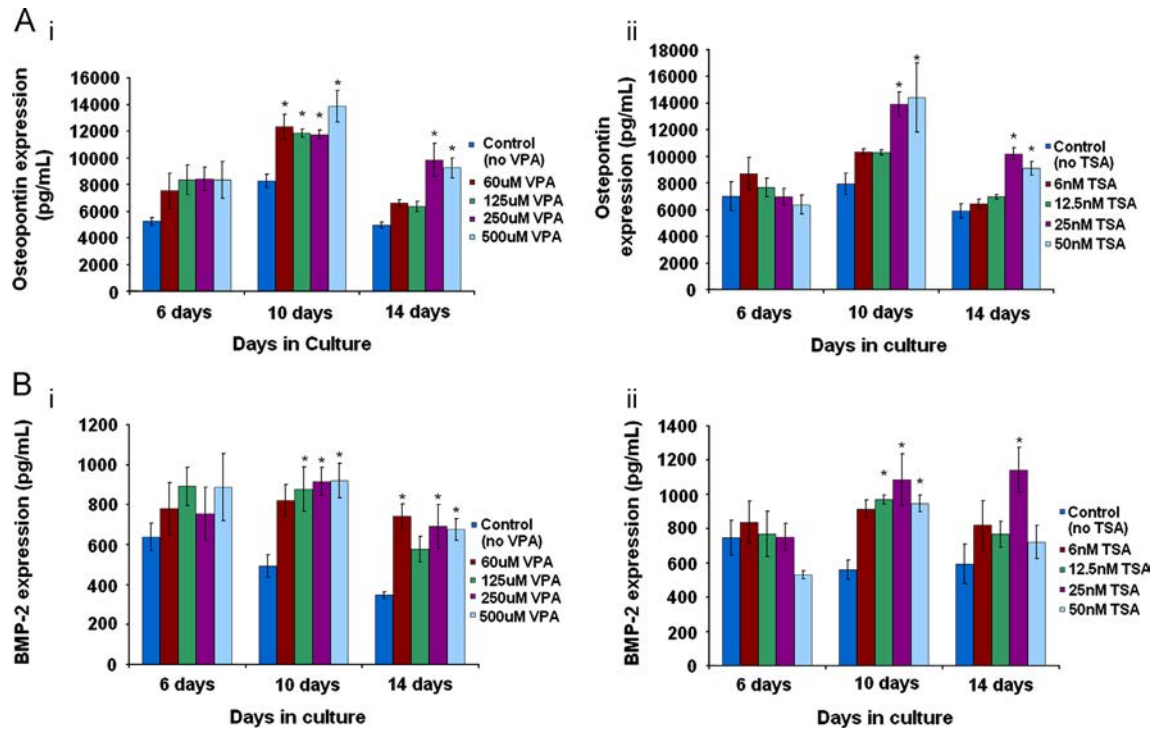




**Fig. 3** – Mineralization per cell analysis in primary DPCs cultured in an HDACi-supplemented mineralization medium. VPA (A) concentrations of 30 µM, 60 µM, 125 µM, 250 µM and 500 µM and TSA (B) concentrations of 3 nM, 6 nM, 12.5 nM, 25 nM and 50 nM supplemented in the mineralization medium for 24 h at the initiation of the experiment prior to culture for a further 13 days in HDACi-free mineralization medium. Mineral production per cell data for VPA (A) and TSA (B) are based on quantification of Alizarin Red S stain extracted from each experimental well and corresponding cell counts. Statistically significant differences ( $p < 0.05$ ) between the experimental and control is marked by an asterisk (\*), while significant differences between 250 µM VPA and all other concentrations of VPA (except 125 µM) are marked by an arrow (^).



**Fig. 4** – sq-RT-PCR demonstrating differential gene expression in primary DPCs for 5 and 10 day cultures with 125 µM VPA or 25 nM TSA. Representative gel images (A) illustrating expression profile of the housekeeping gene GAPDH and the mineralization/dentinogenic-associated markers DMP-1, DSPP, BMP-2, BMP-4 and Nestin. Expression profile of mineralization/dentinogenic-associated markers DMP-1, DSPP, BMP-2, BMP-4 and Nestin relative to control. Volume densities of amplified products were normalized to GAPDH gene expression. Averaged values for each group were calculated and charted as fold-increase of experimental over control band density obtained for each transcript DMP-1 (Bi), DSPP (Bii), BMP-2 (Biii), BMP-4 (Biv) and Nestin (Bv). Statistically significant differences ( $p < 0.05$ ) between the experimental and control are marked by asterisks (\*).



**Fig. 5 – OP and BMP-2 protein expression in primary DPCs cultured in an HDACi-supplemented mineralization medium. VPA concentrations of 60  $\mu$ M, 125  $\mu$ M, 250  $\mu$ M and 500  $\mu$ M and TSA concentrations of 6 nM, 12.5 nM, 25 nM and 50 nM supplemented in the mineralization medium for 24 h at the initiation of the experiment prior to the supernatant being collected at 6, 10 and 14 days and analyzed by ELISA for OP (Ai and Aii) and BMP-2 expression (Bi and Bii) (all reported pg/mL). Statistically significant differences ( $p < 0.05$ ) between the experimental and control are marked by asterisks (\*).**

significantly increased in response to all concentrations of VPA except 125  $\mu$ M ( $p=0.193$ ) and at 25 nM and 50 nM TSA ( $p=0.0001$ ,  $p=0.02$ ), while increased OP protein release was evident at 250/500  $\mu$ M VPA ( $p=0.004/p=0.009$ ) and 25 nM TSA ( $p=0.040$ ).

## Discussion

This study highlights the potential for HDACi in promoting reparative events in the dental pulp and supports the original hypothesis that the two HDACi investigated did not induce significant anti-proliferative, apoptotic or necrotic effects, but did stimulate *in vitro* mineralization. Notably, the increased mineralization was dose-dependent, which is consistent with previous studies utilizing osteoblast [27] and dental-papilla derived cell lines [15,16]. However, this report supports the different responses previously reported between primary and transformed cells following exposure to HDACis [21]. Indeed, mineralization responses in DPCs were significantly induced by concentrations up to 10-fold lower than those previously reported as being active in a transformed papilla-derived cell-line [15]. Nevertheless, the HDACi-induced anti-proliferative effects were observed over similar concentrations in both primary cells and the pulp cell-line, indicating that the concentration ranges at which VPA/TSA stimulate mineral deposition in primary cells were not associated with significant growth inhibition. HDACi-induced mineralization after 14 days ( $\leq 3$ -fold increase compared with control) was evident in cultures initially supplemented with VPA or TSA for 24 h, prior to culture for a further 13 days in a HDACi-free mineralization medium (Fig. 3).

Interestingly, extension of the initial HDACi supplemented culture period (1–7 days) did not further enhance mineral deposition at 14 days (data not shown). Furthermore, up-regulation of the mineralization and odontoblast-associated transcripts was also detected at 5 and 10 days, which are consistent with a previous report indicating that an initial transient dose of HDACi sustains a pro-differentiation effect [27], which is likely due to heritable epigenetic changes [27]. Concomitantly, analyses of mineralization-associated protein expression at 10 and 14 days further highlights VPA/TSA-induced differentiation and corroborates previous reports of HDACi-induced expression of these proteins in mineralizing cells [27,28].

The current study is the first to use primary rat DPCs to investigate the effect of HDACi on cell differentiation and behavior. A range of experimental cell models have previously been used to analyze *in vitro* mineralization including transformed cell-lines and primary cells from rat, mouse, bovine, ovine and human origin [29]. Recent pulp research has utilized the developmentally derived cell-lines MDPC-23 and OD-21 [30], while primary pulp cell models have generally been of human and rat origin, with mouse, bovine and ovine cells used less frequently [31]. Transformed cell-lines possess certain advantages for use in the early stages of drug development as they provide a relatively easily cultured, homogenous cell population with no ethical barriers to use. However, caution should be exercised in extrapolating results from these models as they have a limited phenotypic expression and can exhibit different cellular behavior compared with normal cells [18]. Human primary cells possess properties more reflective of the *in vivo* situation, making them an appropriate model for translational dental pulp research, however, these cells are highly heterogenous, can be difficult to source in

sufficient volume and cannot easily be standardised with respect to genotype, donor age, gender, site and disease state of the tissue [32]. As a result, human studies can be limited by wide phenotypic variation and exhibit a lack of homogeneity in responses, necessitating a significant increase in the number of independent biological samples required. A rat cell model addresses many of the shortcomings of human cell use, with gender, age, weight and pulpal inflammatory state all readily controlled as well as providing a more reliably accessible cell source. It is, however, acknowledged that the mineralization processes in rat primary cells can be influenced by donor sex or age [33] and the continuous growth pattern exhibited in rat's incisor teeth throughout life, in contrast to the limited growth of human teeth [34]. As species-related differences exist between rat and human cells, care should be exercised in extrapolating *in vitro* data to the clinic setting [29]. However, primary rat cells are reported to provide a predictable and consistent model for primary cell epigenetic research [5].

In addition to the induction of differentiation, other cellular processes have been reported to be modulated by HDACi including proliferation and these processes have been successfully targeted for cancer treatment [1,5]. Notably, the mechanism of growth inhibition in transformed cells is linked to cellular induction of the cyclin-dependent-kinase inhibitor p21 [25], either p53-dependently or independently [26]. Currently, there are relatively few primary cell studies which have investigated HDACi-induced expression of p21/p53 and in this study, growth inhibition was evident at the higher concentrations of VPA/TSA applied with significant increases in p53 in response to VPA concomitantly detected. This relationship is somewhat expected as it has previously been reported that the HDACi-concentrations required for anti-proliferative effects in cancer studies are higher than those required for pro-mineralization and anti-inflammatory responses [5,7]. Recently, the sensitivity of the cells to p53-acetylation has been reported as being multi-factorial, being influenced by cell-type, HDACi and the time-point investigated [35]. Specifically, it was identified that the VPA/TSA-mediated p53 effect was evident at 12 h, but not at 24 h. In addition, induction of p53 expression required relatively high concentrations of VPA exposure compared with TSA, and the authors attributed this to possible variations in HDACi selectivity [35]. In the current study, the observed increase in p53 acetylation was also time-dependent, as no altered expression was evident at 24 h, only 48 h. The increase was only evident for VPA, and not TSA, however, this is likely to be dose-dependent and may well have been evident had we used exposure concentrations above those used in this study. This analysis were not however performed in the current investigation as this study aimed to determine the p53-response in DPCs over a therapeutic range induced by relatively low concentrations of HDACis. Therefore, a likely explanation for VPA (but not TSA) eliciting p-53 expression is related to the HDACi concentration used, in addition to the selectivity of VPA compared with TSA.

However, no significant increases in p21 levels were observed with either HDACi applied. This may reflect variable kinetics of anti-proliferative pathways, or that p21 is only stimulated in the pulp cell line [15] and not in primary DPCs, or simply that p21 and p53 expression and activity are independent [35]. To date, there has been no pulpal research with regard to HDACi-induced p21/p53 expression, however, it does appear, that at least for VPA, DPC growth inhibition likely occurs via a p53-dependent pathway.

Notably, no observed change in apoptosis, caspase-3 expression or viability occurred at any HDACi concentration supporting a resistance

to this toxicity in the primary cells used here, which contrasts with the toxicity previously reported using transformed cell-lines [17,18]. It has been proposed that HDACi-mediated resistance may be due to the absence of ROS accumulation and caspase activation in primary cells, which is evident in transformed cells [15,19,36]. The basis of this selectivity may be related to the increased levels of intracellular thioredoxin, which stimulate ROS scavengers and ribonucleotide reductases in normal cells but not in transformed cells [17]. Additionally, it has been proposed that while primary cells possess the capacity to repair HDACi-induced DNA double-strand breaks, transformed cells are less able to do so and this may also contribute to the differences in sensitivity observed [18,37]. Evidently, the HDACi-mediated responses are different in DPCs and pulpal cell-lines and this emphasizes the need to target further experimentation to primary pulp cells as transformed cell-line data may have limited clinical relevance.

No loss of vitality was observed at any HDACi concentration applied and a significant increase in the viability of DPCs was observed following application of all concentrations of TSA compared with control cultures (Fig. 1D and E). These data corroborate previous reports using primary osteoblasts, which identified a transient increase in cell viability in HDACi-treated cultures [4]. Interestingly, a similar trend of VPA-induced increased viability was noted by FC analysis, while not statistically significant, the mean percentage increase in viability between the TSA and VPA treated samples was similar (2.7–5.3% viability increase for VPA; 4.6–6.4% for TSA) highlighting a common trend. A similar HDACi-induced increase in pulp cell viability could potentially direct future research relating to the ability of HDACi to protect pulp cells from toxic effects of dental restorative materials, similar to work previously undertaken using anti-oxidants [9,38,39].

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## Conclusions

This study demonstrated that short-term application of two HDACi to primary DPCs dose-dependently promotes epigenetically-related mineralization and differentiation events at concentrations 10-fold lower those previously described for transformed cells. In contrast to transformed cells, mineralization is stimulated without a significant loss of cell viability and increase in apoptosis is suggestive of DPC resistance to HDACi-mediated toxicity. Notably, for future translational studies the promotion of tissue-repair processes was evident after a single-dose of topical HDACi, thereby supporting the potential benefits of short duration, low concentration HDACi application for vital pulp treatment.

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## CHAPTER 6

### PUBLICATION 5: ORIGINAL SCIENTIFIC MANUSCRIPT

**Duncan HF, Smith AJ, Fleming GJP, Partridge NC, Shimizu E, Moran GP, Cooper PR. (2016). The histone-deacetylase-inhibitor suberoylanilide hydroxamic acid promotes dental pulp repair mechanisms through modulation of matrix metalloproteinase-13 activity. *J Cell Physiol* 231:798-816.**

It has been previously demonstrated that HDACi promoted primary DPC mineralisation at significantly lower concentrations than transformed cells, without an associated loss of cell viability or an increase in cell apoptosis (Chapter 5). However, elucidation of the mechanisms driving the regenerative events remained unresolved. In this study, it was hypothesised that the mechanism modulating the regenerative properties of one clinically-approved HDACi (SAHA) related to transcriptional changes which affected selected mineralisation-associated pathways. The initial element of this study aimed to identify the regulators by which SAHA promoted regenerative processes in primary DPCs using a combination of gene expression microarray, pathway analysis, and molecular validation. Thereafter, pharmacological inhibition of an HDACi-induced novel transcript was investigated using mineralisation, RT-PCR, protein expression and cell migration assays to confirm its role in SAHA-modulated DPC repair.



The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every sale, purchase, and payment must be properly documented to ensure the integrity of the financial statements. This includes recording the date, amount, and purpose of each transaction.

Secondly, the document highlights the need for regular reconciliation of bank accounts. By comparing the company's records with the bank statements, any discrepancies can be identified and corrected promptly. This process helps to prevent errors and ensures that the cash balance is always up-to-date.

Another key aspect is the proper classification of expenses. It is crucial to distinguish between personal and business expenses to avoid any potential tax issues. Business expenses should be clearly identified and supported by receipts or invoices.

The document also addresses the importance of timely payment of bills and taxes. Delaying payments can lead to penalties and interest charges, which can significantly impact the company's cash flow. Therefore, it is recommended to establish a schedule for reviewing and paying all obligations.

Finally, the document stresses the value of seeking professional advice. A qualified accountant or tax advisor can provide valuable insights and ensure that the company's financial practices are in compliance with all relevant laws and regulations.





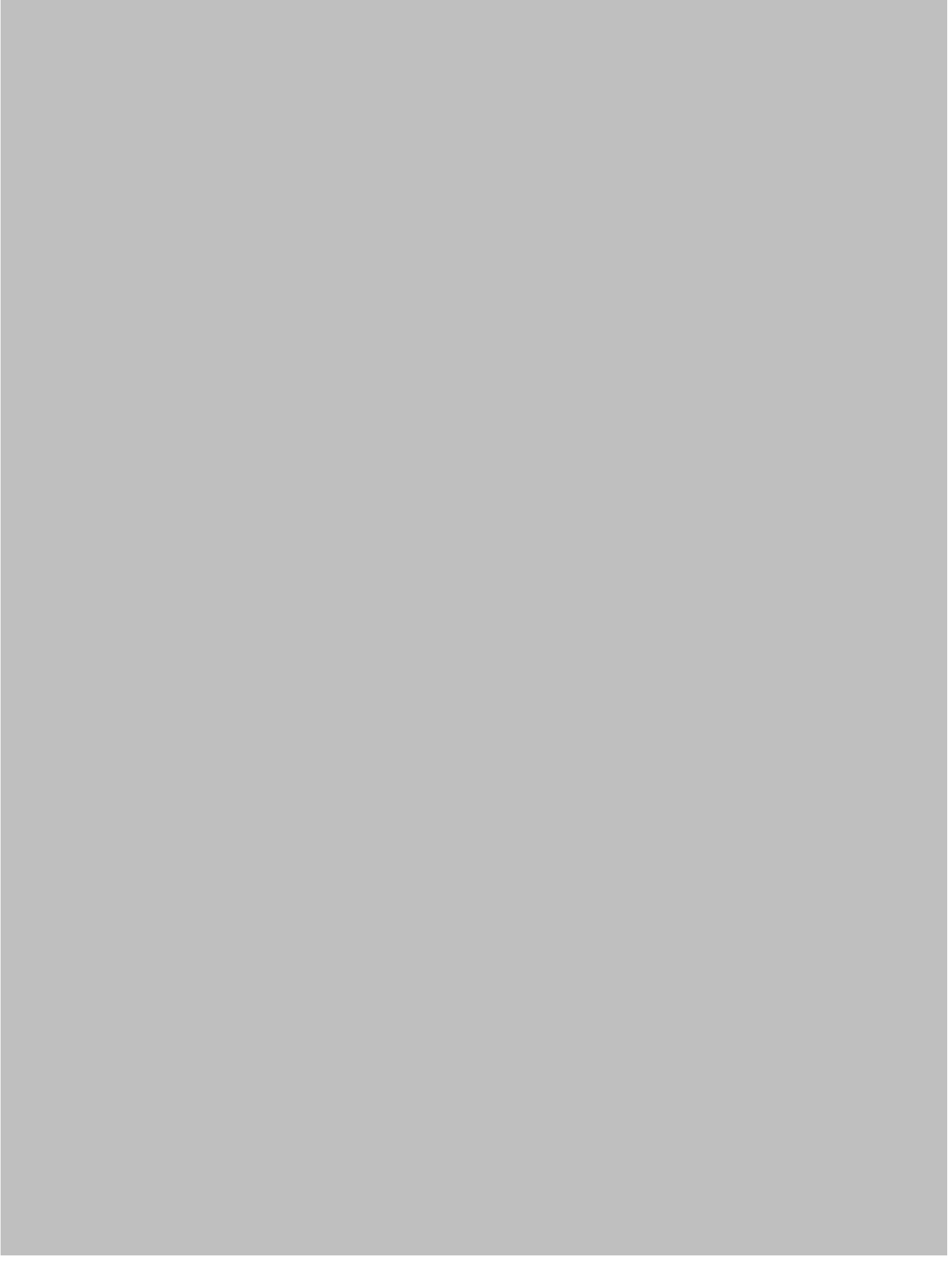
The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every entry, no matter how small, should be recorded to ensure the integrity of the financial data. This includes not only sales and purchases but also expenses, income, and transfers between accounts. The document provides a detailed list of items that should be tracked, such as bank statements, credit card receipts, and invoices. It also outlines the best practices for organizing these records, such as using a consistent naming convention and keeping them in a secure, accessible location. The second part of the document focuses on the process of reconciling accounts. It explains how to compare the company's internal records with the bank's records to identify any discrepancies. This process is crucial for detecting errors, such as double entries or missing transactions, and for ensuring that the company's books are balanced. The document provides a step-by-step guide to performing a reconciliation, including how to identify and investigate any differences. The final part of the document discusses the importance of regular audits. It explains that audits are necessary to verify the accuracy of the financial records and to ensure that the company is complying with all applicable laws and regulations. The document provides a checklist of items to be audited and offers tips on how to conduct an effective audit. Overall, the document is a comprehensive guide to financial record-keeping and reconciliation, designed to help businesses maintain accurate and reliable financial data.



The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that proper record-keeping is essential for the success of any business and for the protection of the interests of all parties involved. The document then goes on to describe the various methods and techniques used to collect and analyze data, highlighting the importance of using reliable sources and ensuring the accuracy of the information gathered.

The second part of the document focuses on the analysis of the data collected. It discusses the various statistical methods and techniques used to interpret the results, including the use of regression analysis, correlation analysis, and hypothesis testing. The document also discusses the importance of understanding the limitations of the data and the potential for bias or error in the analysis.

The final part of the document discusses the implications of the findings and the recommendations for future research. It emphasizes the need for continued research in this area and the importance of using the findings to inform decision-making and policy development. The document concludes by stating that the information provided is intended to be a helpful guide for anyone interested in this field of study.

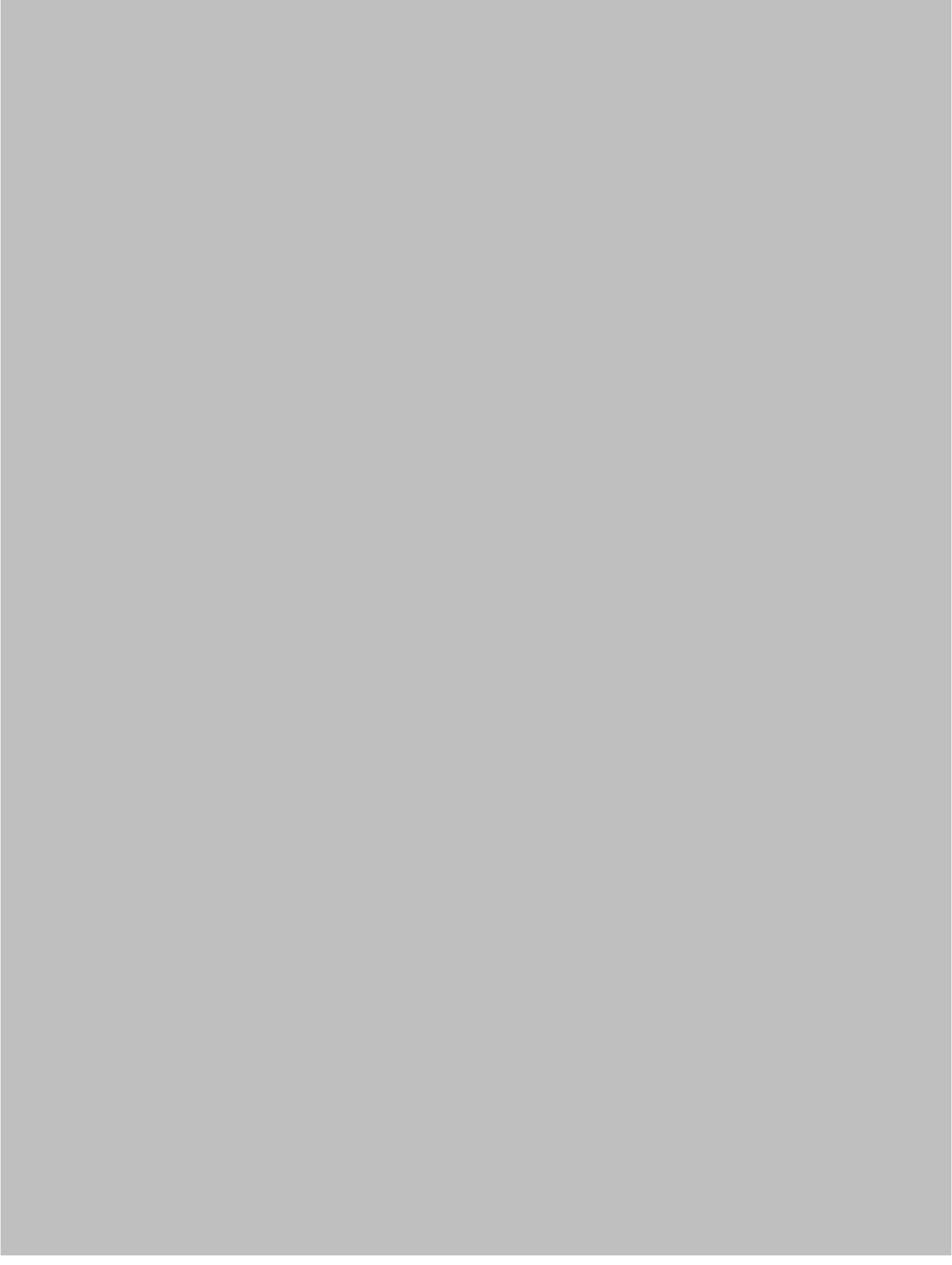


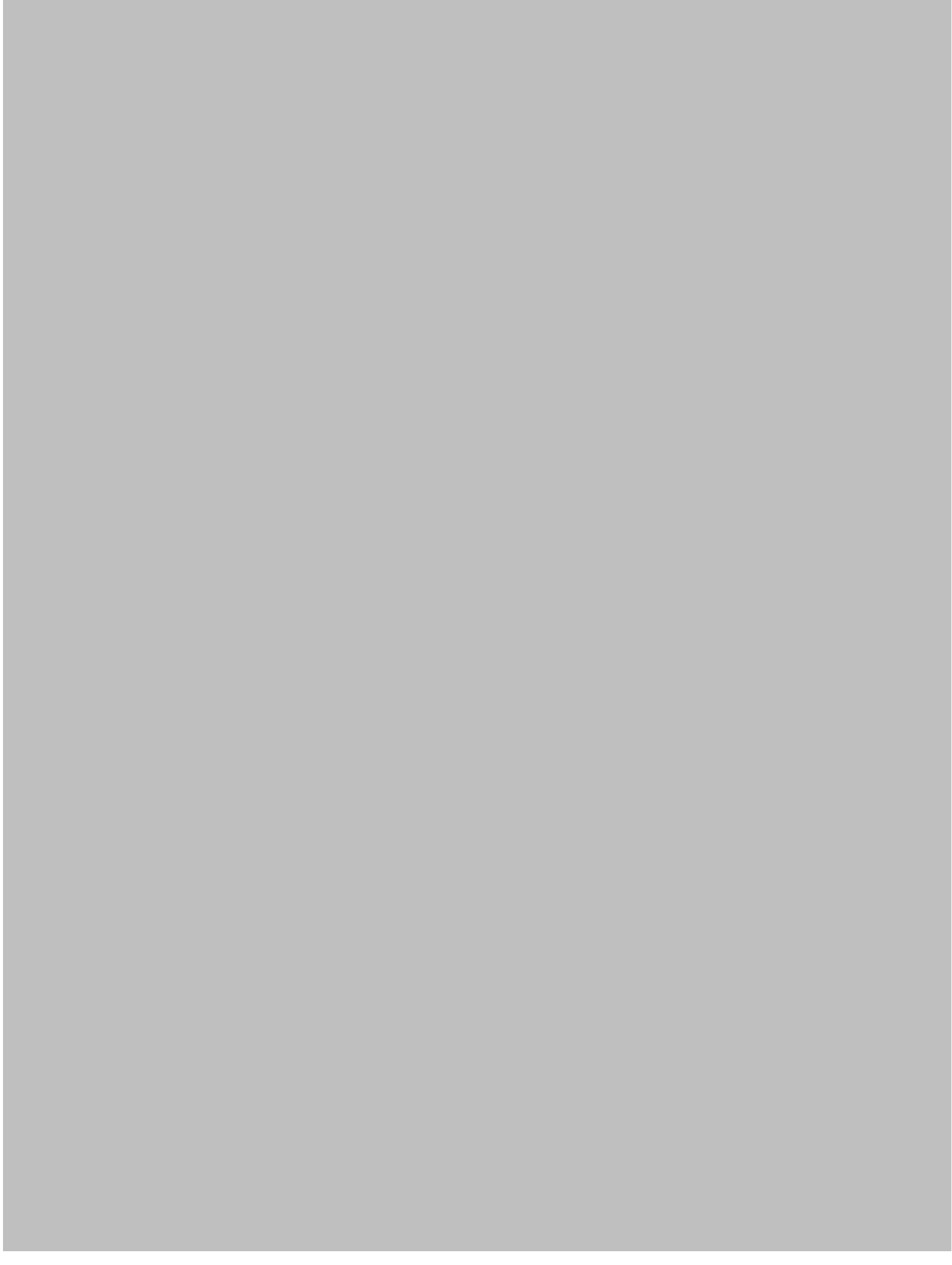
The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every sale, purchase, and payment must be properly documented to ensure the integrity of the financial statements. This includes recording the date, amount, and purpose of each transaction, as well as the names of the parties involved.

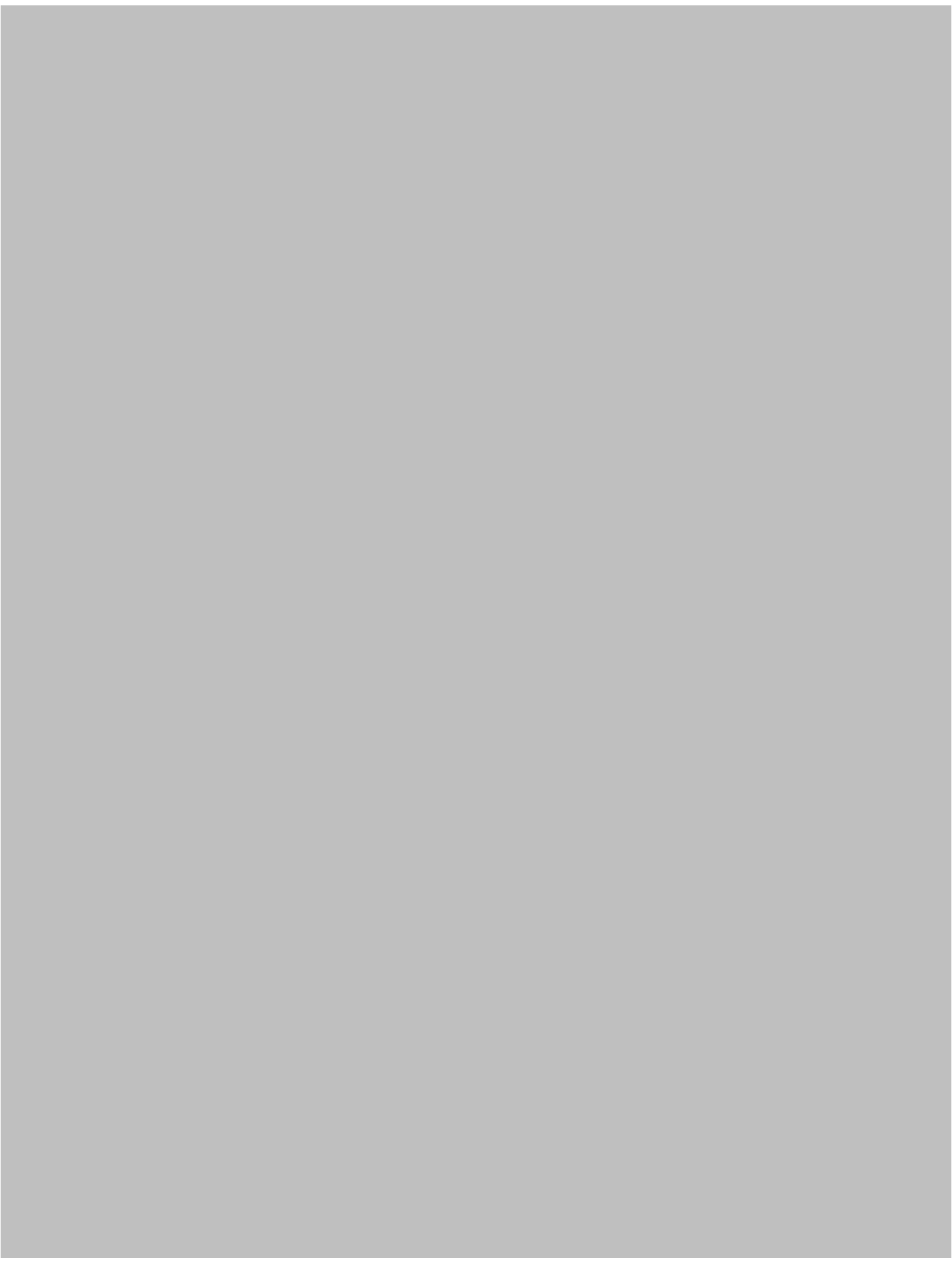
Secondly, the document highlights the need for regular reconciliation of accounts. This process involves comparing the company's internal records with the bank statements to identify any discrepancies. Regular reconciliation helps to detect errors or fraud early on and ensures that the books are balanced at all times.

Thirdly, the document stresses the importance of proper classification of expenses. Expenses should be categorized according to their nature and purpose, such as salaries, rent, utilities, and advertising. This classification is essential for preparing accurate financial statements and for tax reporting purposes.

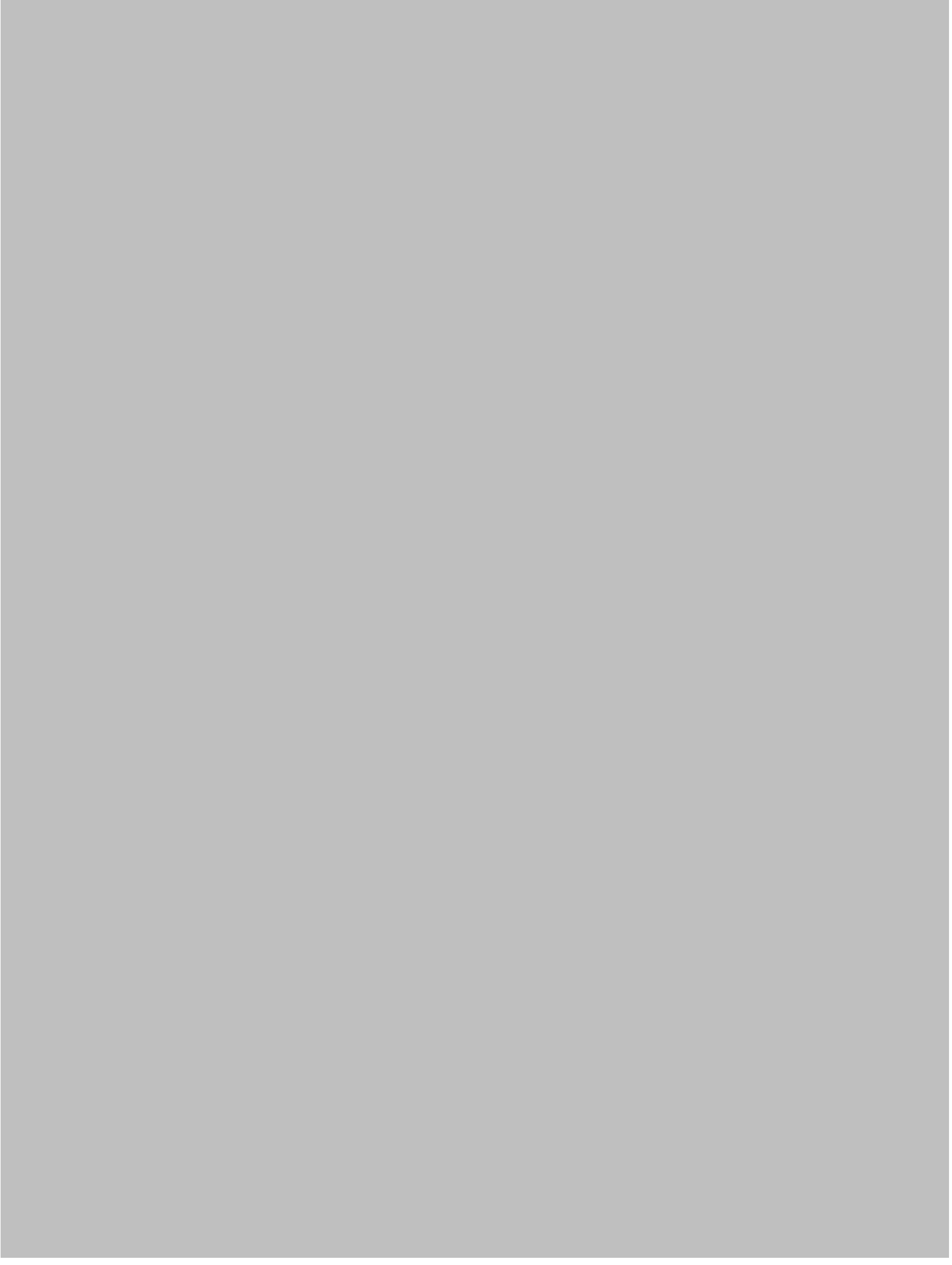
Finally, the document concludes by emphasizing the importance of transparency and accountability in financial reporting. Management should provide clear and concise information to the board of directors and other stakeholders, ensuring that all transactions are properly disclosed and explained.



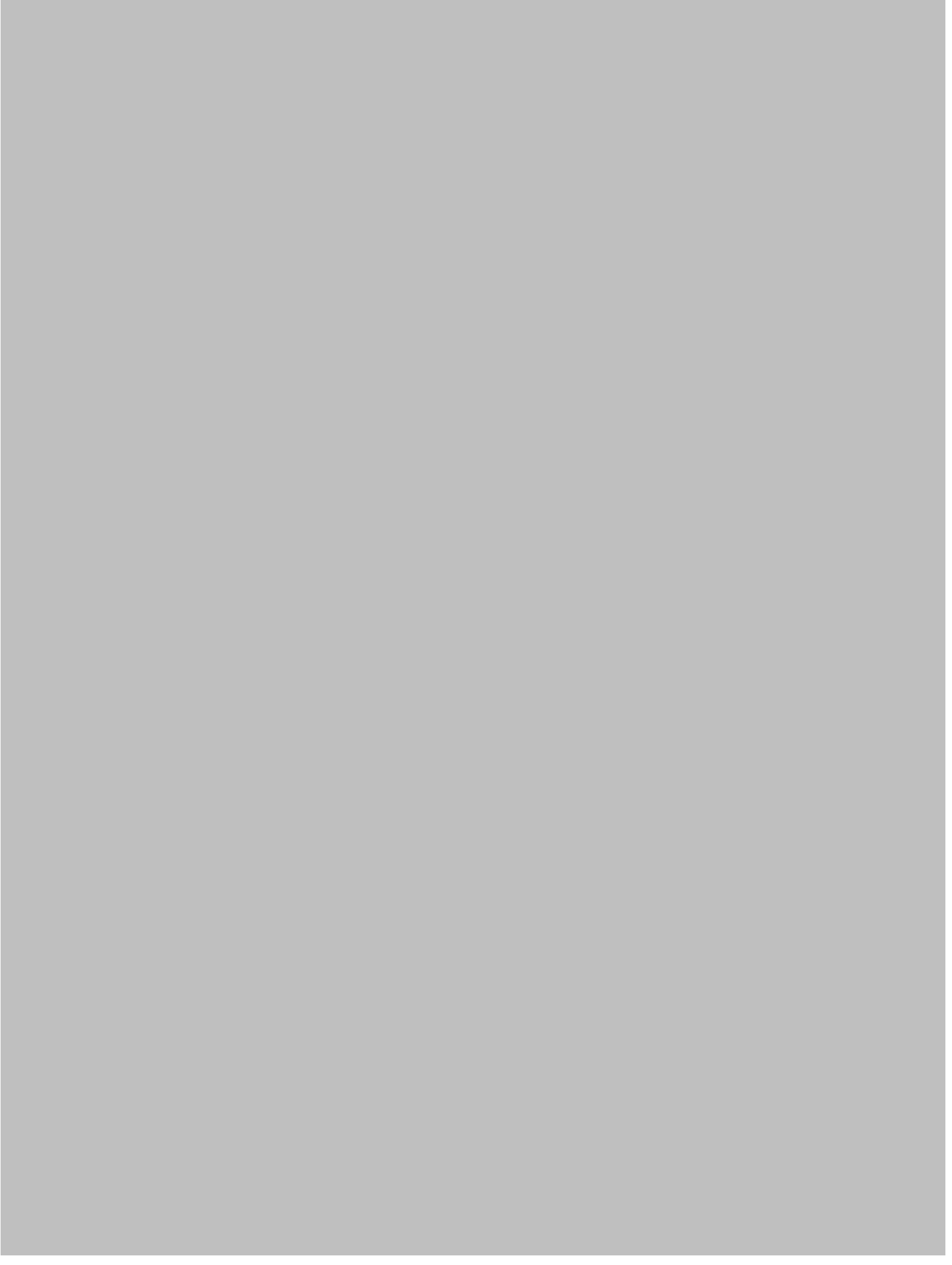




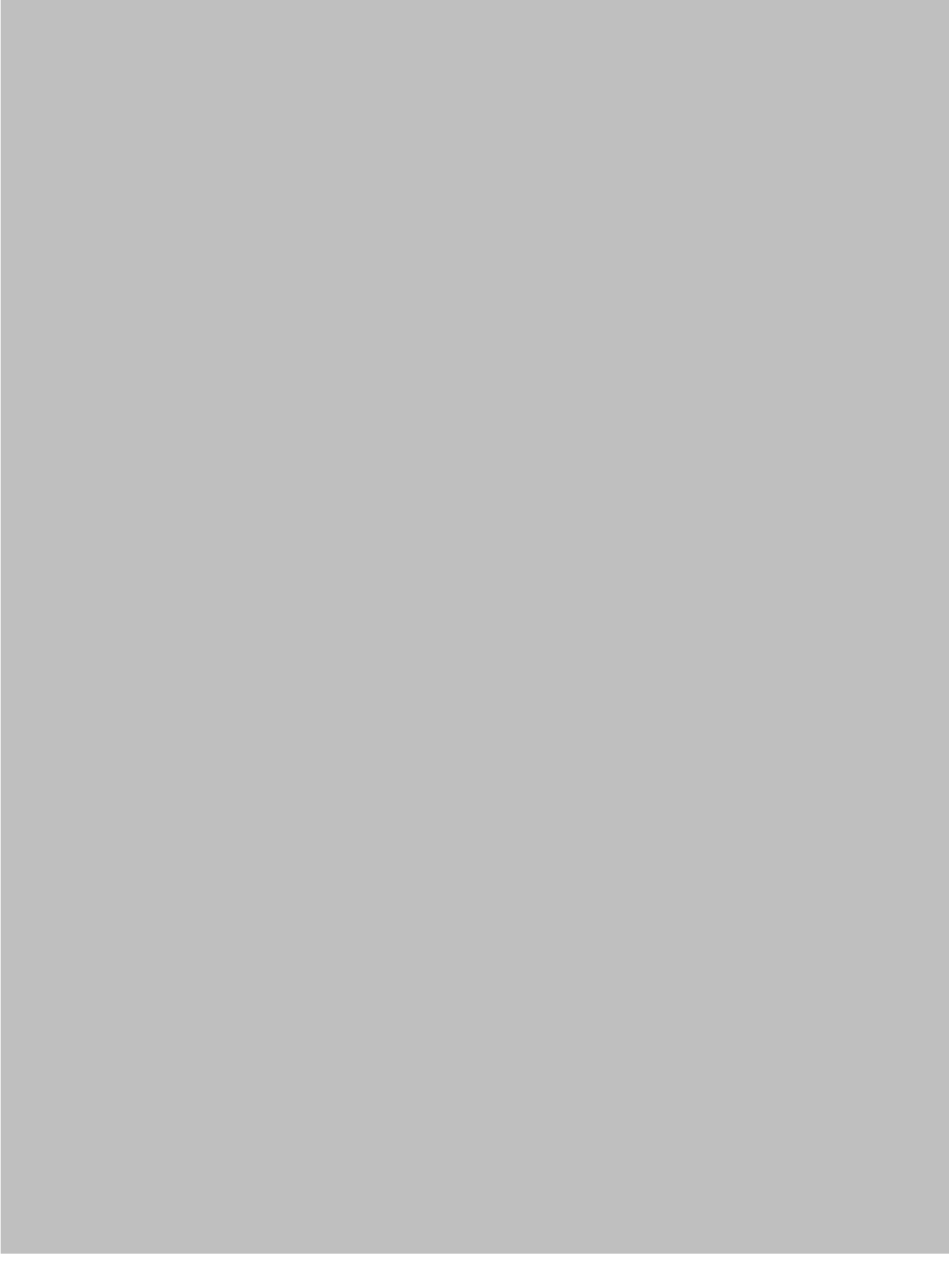




















## CHAPTER 7

### PUBLICATION 6: REVIEW

**Smith AJ, Duncan HF, Diogenes A, Simon S, Cooper PR. (2016). Exploiting the bioactive properties of the dentin-pulp complex in regenerative endodontics. *J Endod* 42:47-56.**

<http://dx.doi.org/10.1016/j.joen.2015.10.019>

Dentine is increasingly regarded as a reservoir of bioactive molecules which can be released into the pulpal environment by restorative procedures, carious processes or dental materials and thereafter, augment reparative/regenerative events in the dentine-pulp complex. The action of released DMCs works in synchrony with the bioactive molecules generated locally by DPCs to promote repair processes. The aim of this study was to review the literature focusing specifically on the role of bioactive molecules located in the dentine-pulp complex and their potential contribution to pulpal reparative events following injury and as a part of regenerative endodontic therapeutic strategies.

# Exploiting the Bioactive Properties of the Dentin-Pulp Complex in Regenerative Endodontics

Anthony J. Smith, PhD,<sup>\*</sup> Henry F. Duncan, BDS, MClin Dent,<sup>†</sup> Anibal Diogenes, DDS, MS, PhD,<sup>‡</sup> Stephane Simon, DDS, PhD,<sup>§</sup> and Paul R. Cooper, PhD<sup>\*</sup>

## Abstract

**Introduction:** The development of regenerative endodontic therapies offers exciting opportunities for future improvements in treatment outcomes. **Methods:** Advances in our understanding of regenerative events at the molecular and cellular levels are helping to underpin development of these therapies, although the various strategies differ in the translational challenges they pose. The identification of a variety of bioactive molecules, including growth factors, cytokines, chemokines, and matrix molecules, sequestered within dentin and dental pulp provides the opportunity to present key signaling molecules promoting reparative and regenerative events after injury. **Results and Conclusions:** The protection of the biological activity of these molecules by mineral in dentin before their release allows a continuing supply of these molecules, while avoiding the short half-life and the non-human origin of exogenous molecules. The ready release of these bioactive molecules by the various tissue preparation agents, medicaments, and materials commonly used in endodontics highlights the opportunities for translational regenerative strategies exploiting these molecules with little change to existing clinical practice. (*J Endod* 2016;42:47–56)

## Key Words

Bioactive molecules, cell signaling, dentin, pulp, regenerative endodontics

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Endodontics is constantly evolving through improvements in our understanding of the basic sciences underpinning disease and its treatment, advances in materials and other technological innovations, together with the accumulation and sharing of clinical experience gained by practitioners. The combination of all of these various factors provides the greatest opportunities for improved clinical outcomes. Biologically based therapies have long been important in endodontics and offer significant promise for future developments. Historically, pulp capping and other approaches to pulpal wound healing can be traced back to at least the 18th century (1). More recently, however, advances in biology have allowed the basis of these approaches to be better understood and for novel, more targeted approaches to be pursued.

The term *regenerative endodontics* can be defined according to one's own perception as to what it encompasses. Perhaps in its least specific definition, it encompasses many of the different biologically based therapies aimed at stimulating pulpal wound healing. The American Association of Endodontists (AAE) defines regenerative endodontics as "biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp dentin complex" (2). This definition clearly states that the aim of these procedures is the replacement of lost cells and root structure without making the claim that this replacement is a complete recapitulation of the once lost tissue. However, a much narrower definition could be used that implies that these procedures must induce the regeneration of new tissue resembling the native pulp-dentin complex at the histologic level with the expected physiological functions; however, this seems an unlikely outcome of current regenerative endodontic approaches (3). Reports of endodontic procedures that use an intracanal antibiotic paste containing metronidazole and ciprofloxacin (double antibiotic paste) (4) or a triple antibiotic paste (5) combined with early investigations of the role of the blood clot in pulpal healing (6) have helped to develop clinical regenerative endodontics in recent years. A variety of chair-side procedures involving creation of a blood clot in a diseased pulp have now been reported and are often described as revascularization procedures (7). These reports have all contributed to a call for action to develop regenerative endodontic therapies for clinical use (2) and initiatives from the AAE to collate experience and support development of regenerative endodontics in clinical practice. There are now several examples of variations of the original clinical revascularization procedures that include the use of other scaffolds such as platelet-rich plasma (8), platelet-rich fibrin (9), and gel foam (10), as well as the application of exogenous growth factors such as fibroblast growth factor 2 (FGF-2) (10). These variations, among other developments, highlight the rapid evolution of the field of regenerative endodontics and the translational nature of its propelling research. Although there is no consensus definition of regenerative endodontics, the exciting future exploitation of this area may be best served by the adoption of a less strict definition for regenerative endodontics and the avoidance of semantic debates. In this way, clinical practice can keep progressing with the application of regenerative procedures that focus on meaningful patient-centered outcomes such as resolution of the disease and tooth survival. However, we recognize complete regeneration of a pulp-dentin complex that resembles the native lost tissue remains the ultimate goal, and that it is the driving force of considerable research efforts that are moving the field of regenerative endodontics into future more sophisticated therapy modalities.

Indeed, the biological focus of the exciting research in regenerative endodontics has greatly advanced our understanding of this area. In this review, we focus specifically

on the involvement of bioactive molecules found in dentin-pulp, their potential contributions to reparative/regenerative events, and their potential clinical exploitation. Although targeting bioactive molecules within dentin-pulp represents only one facet of regenerative endodontics, it may potentially allow rapid progress to be achieved within this area in the short-term with only subtle changes to existing clinical practices.

## Bioactive Properties of Dentin and Pulp

Traditionally, dentin has been regarded as a relatively inert mineralized connective tissue that shows minimal remodeling after formation and in the absence of disease. In contrast, pulp is considered to resemble a classic soft connective tissue in terms of displaying turnover and remodeling, although its more gelatinous consistency contrasts with the fibrous nature of many other connective tissues. Although these perceptions of dentin and pulp have long been clinically prevalent, it is important to recognize that dentin has been known to show bioactive properties for more than 4 decades. Demineralized dentin matrix was demonstrated to induce pulpal repair (11) and apical closure (12) in primates, and this inherent pro-mineralizing effect was reportedly due to bone morphogenetic protein (BMP) activity (13–16). Subsequently, the soluble pool of non-collagenous proteins in dentin, which is released during demineralization of the tissue, was reported to induce both reparative (17) and reactionary dentinogenesis (18, 19). Furthermore, the actions of transforming growth factor- $\beta$  (TGF- $\beta$ ) and other BMP family members on induction of odontoblast terminal differentiation in tooth development could be replicated by preparations of soluble dentin non-collagenous proteins (20). Thus, the perceived inertness of dentin reflects the immobilization and sequestration of these bioactive molecules within the matrix. However, their subsequent dissolution such as during caries provides a local release of their bioactivity.

Earlier studies focused on minimally purified dental tissue preparations, and where characterization of the preparations was undertaken, the analytical techniques were constrained in their ability to

resolve individual bioactive molecules. However, advances in understanding of the nature of these various bioactive molecules and the techniques used for their characterization have since allowed a diverse group of molecules to be identified, many of which were recently reviewed (21). This diverse group of molecules encompasses growth factors, chemokines, cytokines, extracellular matrix molecules, and bioactive peptides, reflecting the complexity of the cellular signaling events capable of being induced. The subsequent discussion of these bioactive molecules and their potential involvement in dentin-pulp regeneration will focus on the signaling involved in the cascade of biological events associated with regeneration rather than simply cataloguing the molecules present. Although considerable research has investigated the biological actions of individual molecules in dentin-pulp regeneration, the microenvironment at sites of tissue injury will reflect the local dissolution of a multitude of bioactive molecules; thus, it is important to note that the summation and indeed synergistic actions of these molecules may differ significantly from those when present individually (22). Although a diverse range of bioactive molecules are also found within the dental pulp, their long-term bioavailability may be constrained by more rapid turnover of the pulpal extracellular matrix and the fact that this source may be unavailable in cases of pulpal necrosis. Thus, dentin can be considered a reservoir of growth factors and other bioactive molecules with important roles in repair and regeneration (Table 1).

The inertness of dentin reflects the immobilization and sequestration or “fossilization” of the bioactive molecules within the matrix. In health, these molecules will largely remain in their “fossilized” state, and it is only when injury and disease occur that matrix dissolution can be observed, leading to local release of these bioactive molecules. This is perhaps an oversimplification because the mechanism of immobilization/association of different bioactive molecules within the dentin matrix varies. In some cases, these molecules are associated with the dentinal mineral phase by a relatively nonspecific, perhaps ionic binding. However, for other molecules, the binding may be more specific in nature (eg, the specific interaction of TGF- $\beta$ 1, although not other

**TABLE 1.** Key Growth Factors and Morphogens Present in Dentin Known to Play Important Roles in Regeneration and Repair

Key growth factors in dentin matrix	Regenerative function
TGF- $\beta$ 1 (23, 24)	Involved in primary odontoblastic differentiation (25, 26) and in promoting tertiary dentinogenesis (20)
TGF- $\beta$ 2 (23)	Its expression is upregulated on differentiation of DPSCs into a mineralizing phenotype (27)
TGF- $\beta$ 3	Promotes odontoblastic differentiation (28, 29)
BMP-2 (30)	Promotes odontoblastic differentiation in both <i>in vitro</i> and <i>in vivo</i> models (31) and the induction of DSPP and increases alkaline phosphatase activity (32)
BMP-4 (30)	Increases odontoblastic differentiation (33)
BMP-7 (34)	Promotes mineralizing phenotype in DPSCs (35, 36)
Insulin growth factor-1 (37, 38)	Promotes proliferation and differentiation of DPSCs and SCAP into a mineralizing phenotype (39, 40)
Hepatocyte growth factor (41)	Promotes migration, proliferation, and survival of MSCs (42)
VEGF (24, 43)	Potent angiogenic factor (44–46) that has been shown to promote blood vessel formation in tooth slices implanted subcutaneously in SCID mice (47)
Adrenomedullin (48, 49)	Promotes odontoblastic differentiation through activation of p38 (22)
FGF-2 (24, 43)	Promotes stem cell homing (chemotaxis), stemness, and angiogenesis (44)
Platelet-derived growth factor (23)	Promotes angiogenesis (50), chemotaxis of MSCs (51), modulates the process of odontoblastic differentiation (52), acting synergistically with other growth factors (53)
Epidermal growth factor (43)	Enhances neurogenic differentiation of DPSCs (54) and SCAP (55)
Placenta growth factor (43)	Promotes angiogenesis (44) and osteogenic differentiation of MSCs (56)
Brian-derived neurotrophic factor (38)	Promotes neuronal growth and axonal targeting (57)
Glial cell line–derived neurotrophic factor (38)	Promotes nerve regeneration <i>in vivo</i> (58) and pulp cell survival/proliferation (59). Increased in expression during odontogenic differentiation (60).
Growth/differentiation factor 15 (38)	Promotes axonal regeneration and function after injury and plays important role in neuronal maintenance (61)

isoforms, with decorin/biglycan in dentin) (62). Thus, dissolution of different bioactive molecules from dentin may occur over a range of conditions that permit modulation of the release of these molecules. Our understanding of the different tissue pools of various molecules in dentin-pulp and their relative solubility is still very limited; however, a better understanding could provide a powerful means to enable release of these molecules to stimulate natural repair processes.

## Biological Events Associated with Regeneration

Repair and regeneration of dentin-pulp comprise a cascade of cellular events with matrigenic, angiogenic, and neurogenic outcomes, reflecting the various processes associated with generation, homeostasis, and function of these tissues. In natural pulpal wound healing, all of these processes are initiated and controlled by a variety of signaling molecules derived from the matrices of dentin-pulp as well as defense, inflammatory, and immune cells associated with disease and its progression. To understand the potential scope of dentin-pulp derived bioactive molecules in repair/regeneration, it is helpful to consider the cascade of cellular processes and events taking place (Fig. 1).

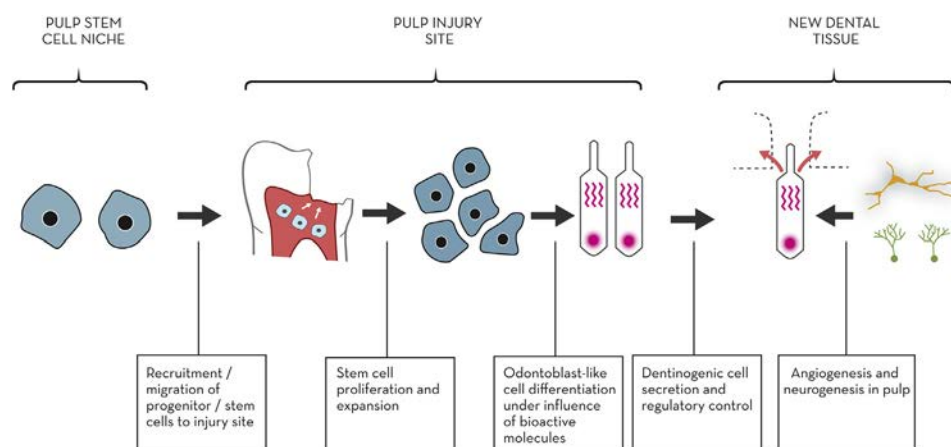
The physiological embryonic development of the dentin-pulp complex is coordinated by an exquisite blueprint of processes under tight temporospatial control (26). Conversely, the postnatal repair and regeneration of these tissues show much less degree of regulation. In part, the less controlled environment of repair and regeneration is a consequence of the exposure and release of a variety of bioactive molecules with potent cellular signaling properties from dentin-pulp in addition to the interplay with the inflammatory process (63). The more limited temporospatial and dosage regulation of signaling molecules, as compared with physiological tooth development, emphasizes the pathologic nature of events occurring during repair/regeneration. This perhaps helps to explain why clinical regeneration of a truly physiological-like pulp tissue is a daunting goal. The complexity of the cellular signaling environment is further exacerbated by the dynamics of the disease process on the release and exposure of bioactive molecules in dentin-pulp and their functional modifications through partial degradation by the acidic and enzymatic activities associated with disease progression (64, 65). Furthermore, surgical intervention by the clinician with endodontic irrigants, disinfectants, medicaments, and other agents will likely also modulate the signaling environment. We will describe how this complex and variable signaling environment may influence cellular events after injury and offers exciting opportunities for development of new therapeutic approaches that exploit the bioactive molecules in dentin-pulp.

Although correlation of specific bioactive molecules with the different signaling steps associated with repair/regeneration is a laudable goal, some of these steps may involve networks of molecules, and also, more than 1 molecule may be able to duplicate the same action for some steps, reflecting either biological redundancy or other nuances of signaling.

## Pulp Cell Niche and Stem Cell Behavior

Stem cells reside within niches, which provide a microenvironment responsible for maintaining the cells in their stem-like, undifferentiated state. This niche is determined by a complex interplay between the stem cells themselves, surrounding cells of various lineages, extracellular matrix, and soluble molecules including growth factors. These niches are generally regarded as providing relatively stable microenvironments, which likely only undergo significant disruption during disease and tissue remodeling. Although the concept of the niche is well-established, our understanding of its nature and behavioral influences on stem cells is still limited. These niches are often described by various markers known to be expressed at these sites, although the functional importance of many of these markers remains unclear. However, Notch signaling has been proposed to be important in controlling stem cell fate in the dental pulp after tooth injury (66). In the dental pulp, a perivascular stem cell niche has been reported (67, 68), which perhaps reflects the transient movement of vasculature-derived stem cells to the pulp. The prominent compartmentalization of stem cells on blood vessels within the apical papilla (69) and inflamed periapical tissues (70, 71) also indicates that perivascular stem cells participate in current regenerative procedures that have been shown to recruit cells from the apical region in both immature (72) and mature teeth (71).

Many of the bioactive molecules present in dentin-pulp have the potential to influence stem cell niches, although our understanding of these interactions is limited. Growth of pulp cells on a layer of isolated pulp extracellular matrix was shown to decrease their proliferation rate and favor expression of a stem cell-like phenotype (73). Furthermore, when these cells were grown in mineralization-inducing conditions, the pulp matrix allowed increased mineralization. Indeed, it has been demonstrated that the “stemness” of mesenchymal stem cells (MSCs) can be prolonged in culture when these cells are cultured on an extracellular matrix that mimics their native niche (74). Collectively, these studies demonstrate that the niche with its rich array of attachment and bioactive molecules has the ability to maintain stem cells at their



**Figure 1.** Schematic of the cascade of biological steps associated with healing events during dentin-pulp regeneration.



maximum differentiation potential. However, there is still much to learn of the cell-matrix interactions controlling pulp stem cell behavior.

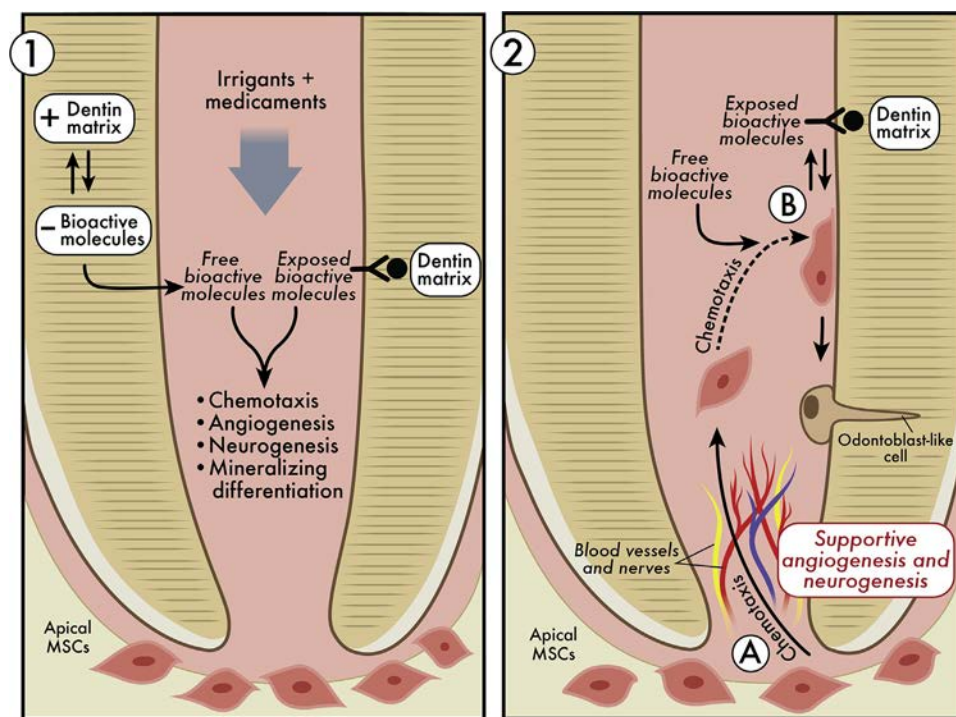
## Pulp Stem/Progenitor Cell Recruitment

Recruitment of stem/progenitor cells from their perivascular or other niches is a critical step in regeneration and guided tissue repair after injury. Release of chemotactic molecules, such as dentin or pulp matrix-derived molecules, at sites of tissue injury may be important to the recruitment process (Fig. 2). Perivascular niches for stem cells are attractive sources for recruitment after carious injury because of the close proximity of much of the pulp vasculature to the odontoblast layer (75, 76). Both dentin and pulp matrices have been reported to contain molecules with chemotactic properties (53, 65, 77), and although some of this activity may be ascribed to growth factors with known cell homing properties (53), other molecules are also likely to be involved. For example, complement activation and generation of C5a has been reported to be one of the molecules associated with lipopolysaccharide-induced pulp progenitor cell recruitment (77).

The target cell specificity of these chemotactic molecules in dentin and pulp is still not well-understood. Dentin matrix proteins can induce recruitment of cells involved in the inflammatory and immune responses of the pulp after injury (78) as well as pulp cells (65), and in the latter report, preferential attraction of cells expressing a range of stem cell markers was observed. Matrix-resident chemotactic molecules provide attractive candidate mediators for stem/progenitor cell recruitment because their release during carious tissue dissolution (18, 79) and irrigants like EDTA (24, 80, 81) used during endodontic treatment will likely lead to chemotactic gradients providing cues for the spatial localization of stem cells along dentinal walls in regenerative endodontic procedures (Fig. 2).

## Pulp Stem/Progenitor Cell Expansion

Little consensus exists as to how many stem/progenitor cells exist within the dental pulp or even how many are required during repair and regeneration after tissue injury. However, it may be expected that the numbers of resident stem cells are relatively small, and some *in situ* or *ex vivo* expansion of their numbers may be required during repair and regeneration. Interestingly, the apical papilla (82) and the inflamed periapical tissues (70) appear to be enriched sources of undifferentiated stem cells that may play an important role in regenerative endodontics, particularly in immature teeth with open apices. Many of the bioactive molecules identified within dentin and pulp show proliferative effects on a variety of cell types including stem cells (73). Some of these activities may be ascribed to the various growth factors within dentin-pulp (15), although other molecules resident in these matrices will likely also contribute to such activities. Although it is valuable to try and catalogue all of the individual bioactive molecules demonstrating *in vitro* proliferative activities on various pulp cell populations in dentin-pulp, caution must be taken in extrapolation of such data to the *in vivo* situation. After injury to the pulp, there will be a complex cascade of cellular events taking place involving an intricate interplay of many bioactive molecules, including inflammatory mediators, with cell signaling properties (83). Anticipation of the outcomes of these interplays of molecules can be very difficult because it is not only the summation of the individual proliferative activities of these molecules that drives the outcomes but also the influence of various autocrine and paracrine regulatory factors in the *in vivo* tissue environment. Attempts to investigate proliferative events *in vivo* during odontoblast-like cell differentiation after tooth injury have clearly demonstrated the active nature of these events (84). However, there is still much to learn of the details of the signaling of these proliferative events and their regulation.



**Figure 2.** Schematic illustrating the potential actions of irrigants and medicaments in the release and/or exposure of bioactive molecules sequestered in dentin and their influences on regenerative events including chemotaxis, odontoblast-like cell differentiation, mineralization, angiogenesis, and neurogenesis.

## Dentinogenic Cell Differentiation

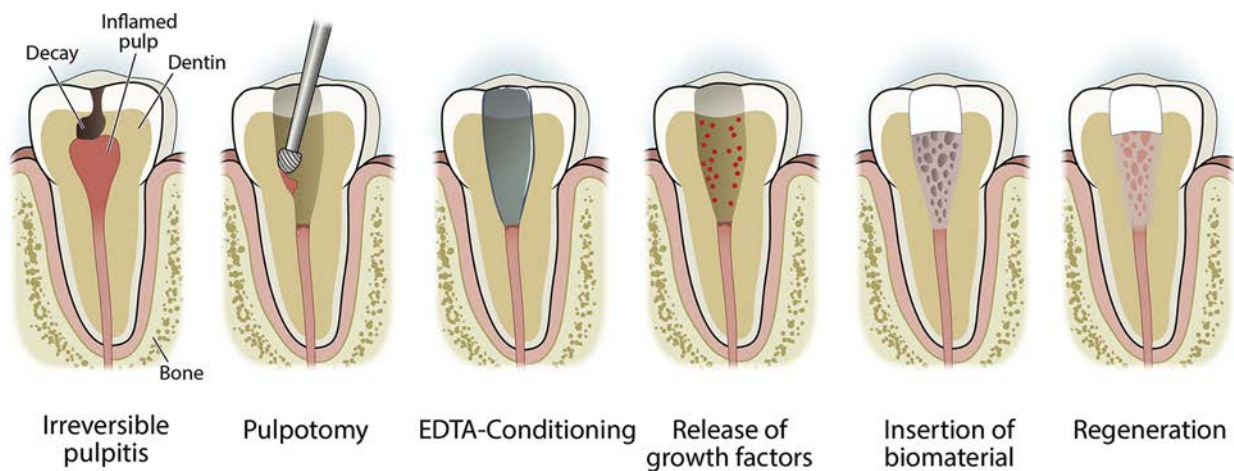
Localized odontoblast cell death often follows moderate to severe dental trauma or carious injuries. Recruitment and differentiation of a new generation of odontoblast-like cells may subsequently ensue, allowing reparative dentinogenesis to occur, such as is seen in situations of mineralized bridge formation after pulp-capping procedures (17).

Differentiation of odontoblast-like cells during reparative dentinogenesis shows a number of parallels with the terminal differentiation of primary odontoblasts during tooth development (25). However, it must be recognized that differentiation of a new generation of odontoblast-like cells is a pathologic rather than physiological event, and the tightly regulated temporospatial control of odontoblast differentiation seen during tooth development is lacking. As a consequence, there may be considerable heterogeneity in the phenotypes of odontoblast-like cells and the matrices they secrete. At the morphologic level, this may be seen as variations in the tubular nature of dentin (85). Also, there appears to be differences in mineral composition of that formed by different MSC populations such as dental pulp stem cells (DPSCs) and stem cells of the apical papilla (SCAP), suggesting also heterogeneity that is based on the cell type involved in the differentiation process (86).

There is a dearth of knowledge relating to the molecular markers identifying “true” odontoblast-like cells from other forms of mineralizing cells such as osteoblasts. Nevertheless, it is important to remember that odontoblast-like cells do not represent a single, well-defined phenotype, and this has crucial implications for their robust identification. Although primary odontoblasts express a profile of molecular markers, including nestin, dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP-1), and matrix extracellular phosphoglycoprotein among other markers, such a profile is not unique to odontoblasts, and relatively simple molecular characterization is not necessarily a robust means of identifying the odontoblast phenotype. The addition of other identification criteria such as cellular morphology and matrix morphology, especially regularity of tubular structure, can increase confidence, although the application of these additional criteria is not necessarily easy. For example, the morphology of an odontoblast varies through its secretory life cycle (87, 88), and the tubular density of dentin will vary at different points of its thickness because of odontoblast crowding as dentinogenesis proceeds in a pulpal direction. Therefore, it is easy to see that the widespread identification of odontoblast-cell differentiation during dentin-pulp repair/regeneration in many different published studies in this field

may not be quite as robust as previously thought. In the majority of these studies, there is little doubt that differentiation of a mineralized cell phenotype occurred, but whether these cells truly share the same phenotype as their physiological counterparts, the primary odontoblasts, remains unresolved. In functional terms, these various odontoblast-like cells and the matrices they secrete likely provide satisfactory outcomes for clinical procedures aimed at restoring the functional integrity of injured dental tissues. However, they cannot be considered to provide true regeneration of physiological-like morphologies for these tissues, and our limited understanding of the factors driving their differentiation means their generation can be somewhat serendipitous.

To try and identify the key morphogenic molecular signals for odontoblast-like cell differentiation, it is useful to look to those events occurring during physiological tooth development. During the late bell stage, inner enamel epithelium-derived growth factor signals, immobilized on the dental basement membrane, are presented to the peripheral cells of the dental papilla, leading to their terminal differentiation to odontoblasts (89). TGF- $\beta$  superfamily members appear to be responsible for signaling odontoblast terminal differentiation physiologically and experimentally, because only immobilized TGF- $\beta$ 1 and TGF- $\beta$ 3 or a combination of FGF-1 and TGF- $\beta$ 1 could stimulate the differentiation of functional odontoblasts over extended areas of the dental papilla and allow for maintenance of gradients of differentiation (20, 90, 91). The ability of preparations of soluble dentin matrix proteins to mimic the effects of these growth factors on experimental embryonic odontoblast differentiation (20) also concurs with the effects of such dentin matrix preparations on odontoblast-like cell differentiation and reparative dentinogenesis in pulp-capping applications (17). The effects of dentin matrix components on the induction of dentinogenesis in pulp-capping situations have long been recognized (11, 17, 92) and may be due to the presence of a range of growth factors in dentin matrix preparations, including members of the TGF- $\beta$  superfamily (14, 23, 37, 41, 43, 93) (Fig. 3). This is supported by reports of tissue-isolated and recombinant growth factors paralleling the actions of dentin matrix preparations (14, 15, 94). However, other components of dentin and pulp may also display morphogenic activity including extracellular matrix molecules such as DMP-1 (95) and BMPs (16, 20). Nonetheless, robust characterization approaches for the phenotype(s) of odontoblast-like cells and experimental functional deletion strategies are required to resolve which components



**Figure 3.** Illustration of the steps associated with clinical management of a tooth with irreversible pulpitis and the potential release of growth factors and other molecules leading to regeneration.

of dentin matrix preparations are essential for signaling odontoblast-like cell differentiation and to what extent the resultant cells resemble physiological primary odontoblasts.

### Dentinogenic Cell Secretion and Its Control

Focus is generally centered on cell differentiation during repair/regeneration in dentin-pulp, but upregulation and control of subsequent secretion by the differentiated cell are also important. Control of dentin secretion occurs physiologically, with downregulation after completion of primary dentinogenesis and upregulation again occurring during episodes of tertiary dentinogenesis. In the absence of regulation of odontoblast secretion, pulp canal obliteration can ensue, with significant implications for both tooth vitality and endodontic treatment (96). Members of the TGF- $\beta$  superfamily are capable of upregulating odontoblast matrix secretion under both physiological conditions (20) and in an *in vitro* repair/regeneration model (93). The target for this cell signaling appears to be activation of the mitogen-activated protein kinase pathway through p38 phosphorylation (22). Identification of this molecular switch for odontoblast secretory activity offers exciting opportunities to clinically modulate such activity through combinations of local targeting with growth factor stimulation or pharmacologic manipulation leading to a more controlled rate of mineral deposition.

### Angiogenesis and Neurogenesis

Both the rich vasculature and a well-developed neural network in the pulp are important for its regeneration and function. After injury or chemical dentin conditioning, various proangiogenic growth factors sequestered in dentin (43) may be mobilized. Their proangiogenic activities (97, 98) may well contribute to local increases in vasculature to support reparative/regenerative events (Figs. 1 and 2). It is well-accepted that angiogenesis is crucial for regenerative procedures because despite being well-equipped to thrive in hypoxic environments (55), stem cells require adequate nutrient supply and gaseous exchange, particularly when in high metabolically demand secretory phases post-differentiation (99). Histologic evidence from both animal models (100–102) and clinical cases of revascularization procedures (103, 104) demonstrates that a good blood supply appears to be present after these procedures. This could be due to the angiogenic factors present on the dentinal matrix and released after the use of EDTA in revascularization procedures (24). In addition, MSCs when found in the hypoxic root canal system can release enhanced concentrations of angiogenic factors such as vascular endothelial growth factor (VEGF) (45, 105). Therefore, current procedures are known to promote robust angiogenesis and are likely driven by locally released growth factors from dentin and recruited stem cells.

The human dental pulp is richly innervated by primarily nociceptors, including neurons expressing potent neuropeptides such as calcitonin gene-related peptide, substance P, and neuropeptide Y (106). On pulpal injury, there is significant neuronal sprouting to the area of injury and repair (107). This enriched innervation is suggestive of a modulatory effect of neurons on dentin-pulp repair. Indeed, re-innervation coincided with pulpal healing and tertiary dentin formation in a model of pulpal repair after replantation of teeth (108), and denervation via nerve transection or pharmacologic ablation results in aberrant dentin formation (109, 110).

Although our understanding of neurogenic events after injury and regenerative endodontic procedures is more limited, the variety of neuropeptides and neurotrophic molecules expressed by odontoblasts and surrounding fibroblasts appear to play a role in the recruitment of nearby free nerve terminals to the area of tissue repair/regeneration

(106, 111–114). In addition to resident pulpal cells, recruited MSCs such as SCAP have been shown to mediate axonal sprouting and targeting through a brain-derived growth factor mechanism (57). Interestingly, in a dog model of regenerative endodontics, a subpopulation of DPSCs with increased expression of brain-derived neurotrophic factor was transplanted into empty root canals, resulting in the formation of an innervated pulp-like tissue (115, 116). Therefore, adequate innervation should be a goal of regenerative endodontics because neurons mediate protective nociception and the modulation of homeostasis processes such as inflammatory responses and odontoblastic function.

### Infection, Inflammation, and Regeneration

As the local environment of the tooth changes and becomes more anaerobic, the carious bacterial biofilm composition becomes more complex as it drives through the enamel, dentin, and pulp (117). The dentin-pulp complex aims to defend itself from the bacterial onslaught, and in the early stages of dental tissue infection, the odontoblasts detect and respond to the microbial presence (118, 119). As the infection progresses, cells more centrally located within the pulp, including resident immune cells, pulpal fibroblasts, vascular endothelial cells, and even stem cells, are also able to mount a response aimed at containing the infection (120–122). A variety of microbial sensors have been described as being present on these cell types, and the best characterized are the Toll-like receptor family that can detect microbial components ranging from their nucleic acids to cell wall constituents (122). After detection of the infection by host cells, cytokines such as interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , tumor necrosis factor alpha, IL-4, IL-6, IL-8, and IL-10 are secreted, and depending on levels and temporality, they can lead to several cellular and tissue outcomes (123, 124). At the early stages and/or at relatively low levels of infection, the inflammatory response may be sufficient to not only contain and remove the infection via the local release of antimicrobial peptides and reactive oxygen species (ROS) but may also stimulate dental tissue repair responses. Indeed, there is now significant evidence to indicate that cytokines and other proinflammatory mediators such as ROS can directly stimulate dental tissue repair responses. However, if the infection increases, there will be an elevation of the inflammatory response mediated by the cytokine signaling network generated from the local cells and from the demineralized dentin that also releases signaling molecules into the milieu (83). Combined, this will lead to increased recruitment of a range of inflammatory cells, including neutrophils, macrophages, T cells, B cells, and plasma cells, which aim to use their intracellular and extracellular armamentarium to contain and kill the invading bacteria (125–128). This localized protective response to tissue infection and injury aims to prepare the tissue for eventual repair, and ideally the inflammatory reaction should be short-lived. Indeed, if it is excessive or prolonged, it will likely result in tissue damage because of either the direct toxic effect of the bacteria or the exuberant host response that results in collateral tissue damage in its attempts to combat the infection.

Central to both the inflammatory and healing responses is the role of the vasculature and the induction of angiogenesis. As has previously been described, angiogenic events can be triggered by the release of potent signaling molecules from the demineralized dentin. Interestingly, many cytokines classically regarded as being fundamental to the inflammatory response, such as IL-1, IL-6, and IL-8, are also proangiogenic (129–131). This pleiotropism is potentially not surprising because of the need for a robust vascular response to enable the delivery of the immune cells to the dental tissue and the removal of unwanted material such as toxic molecules and metabolites. Indeed, the first 3



of the 4 cardinal signs of inflammation—redness, warmth, swelling, and pain—are attributable to the vascular response. To empower the immune and repair responses within the vasculature, adhesion of immune cells and platelets is activated, as is coagulation, thrombosis, and vascular permeability. These processes enable delivery of inflammatory cells to the diseased tissue while isolating the area and setting the stage for tissue repair and regeneration (132). Furthermore, a variety of MSC niches are reported locally within the dental pulp (eg, perivascularly), and clearly MSC mobilization and homing are highly dependent on the vascular and angiogenic responses. Further highlighting the importance of the angiogenic response in dental tissue defense and repair are the data demonstrating the ability of dental MSCs to robustly differentiate down endothelial cell lineages (45, 46, 133). Once recruited, dental MSCs not only have repair characteristics, but they are also immunomodulatory. Their properties include their ability to suppress proinflammatory cytokine levels while upregulating anti-inflammatory molecules, along with their ability to directly temper immune cell behavior (134, 135). It is also of significance that several chemokines and homing molecules are shared by immune cells and MSCs, indicating the importance of the crosstalk between these 2 arms of the tissue defense and repair responses (77, 136) (Fig. 4).

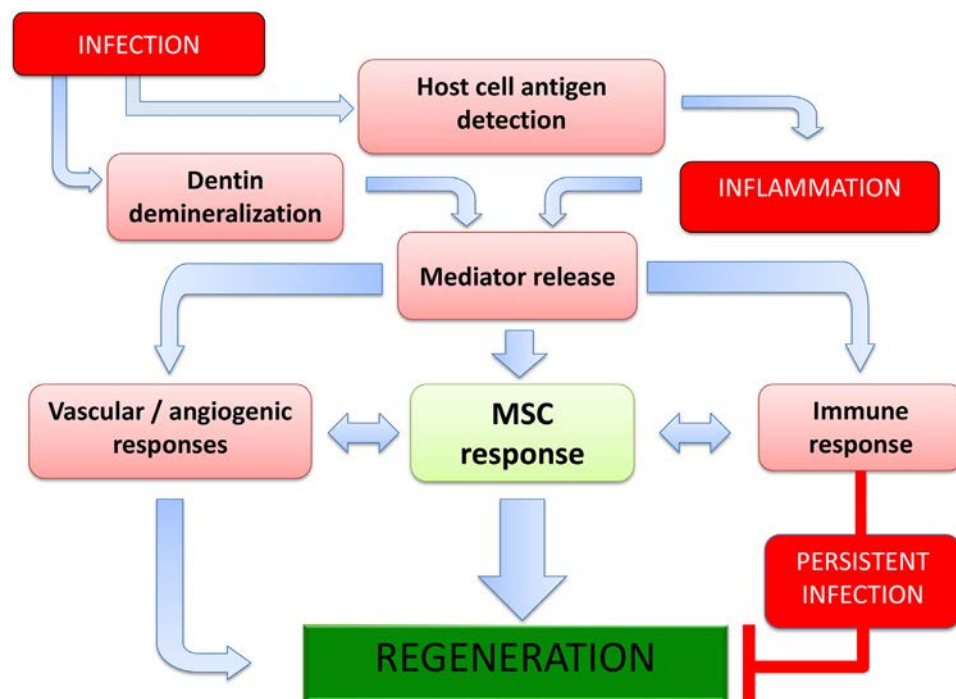
Data from a variety of fields, in particular in mineralized tissue wound healing, are now highlighting the interdependency between inflammation, angiogenesis, and regeneration. It appears that although low-grade or the early inflammatory response can promote tissue repair, as might be seen beneath calcium hydroxide–based or mineral trioxide restorations within the pulp (137–139), relatively high-grade chronic inflammation negatively impacts directly on MSC function and dentinogenic repair and can also delay angiogenesis (83, 140).

Translationaly, the progressive influence of pulpal inflammation on healing creates significant diagnostic responsibility when planning

regenerative procedures to select cases that are likely to respond to treatment. At present, pulpal inflammation is simply classified as reversible, symptomatic, or asymptomatic irreversible pulpitis (141), with the diagnosis established after pain history, clinical/radiographic examination, and pulpal sensibility tests. Traditionally it was believed that the results did not accurately reflect the true histopathologic status of the pulp (142–144) and that new molecular diagnostic test would be required for accurate diagnosis (145). Recently this has been questioned, and a strong correlation has been shown between symptomatology, clinical findings, and the histologic classification of pulp disease (146). However, to improve clinical outcome a better understanding of the interplay between the reparative processes at the molecular level and clinical signs and symptoms may enable the development of new therapeutic modalities for regenerative endodontics.

## Exploiting Bioactive Molecules in Dentin-Pulp

Therapeutic application of a variety of bioactive molecules, whether of natural or synthetic origin, to the injured pulp to promote repair and regeneration has been proposed (147). Although such a strategy is attractive in that it parallels the well-established techniques used in pulp capping, a number of delivery and technical considerations must be addressed. These particularly focus on the preservation of biological activity in a group of labile molecules, which presents significant challenges for routine clinical use where their storage and delivery may be challenging. An alternative strategy is to exploit the bioactive molecules naturally sequestered within dentin. Those within dentin are protected while the tissue is in its mineralized state, and their local release at sites of injury avoids many of the problems of effective clinical delivery in the surgery. Some release of these molecules may occur as a consequence of carious demineralization of the tissue and has the potential to participate in natural regenerative events. However, more targeted



**Figure 4.** Schematic of potential steps and interactions between dental tissue infection, inflammation, vascular responses, and regeneration. An inflammatory response occurs generally after carious bacterial infection, leading to mediator release from host cells and demineralized dentin. This cocktail of cytokines, growth factors, and other signaling molecules acts on MSCs, immune cells, and the vascular system. There is significant crosstalk between these systems, and ultimately if the immune cell response is able to contain or remove the infection, potentially with the involvement of clinical intervention, then dental tissue regeneration can occur.



release might be achieved through local application of demineralizing agents, a number of which are commonly used for tissue preparation during endodontic procedures. Several studies have now highlighted the potential of various agents, epigenetic modifiers, medicaments, and materials to release and expose bioactive molecules in dentin to influence stem cell behavior (24, 49, 148–153). It is noteworthy that initial revascularization-like procedures were designed with the goal of maximum disinfection without taking into account the effect of the chemical debridement on the availability of bioactive molecules present in dentin and the survival and differentiation of stem cells. There is increasing evidence that disinfection can be achieved while promoting a microenvironment that is more suitable for regeneration. Thus, the strategy of targeting endogenous bioactive molecules sequestered in dentin can be achieved with minimal change to current endodontic practice while retaining the fundamental need for adequate disinfection, a pre-requirement for regeneration/repair.

## Concluding Remarks

The future for regenerative endodontics offers exciting promise. In the long-term it is envisaged that regrowth of entire tooth structures within patients may be achievable; however, more short-term goals along that clinical and research journey are now becoming more realistic. Indeed, our increasing understanding of the molecules involved and cell behavior necessary for dentin-pulp complex repair will provide new therapeutic avenues for exploitation. These approaches may well be underpinned by the generation of biomimetic environments that harness the tooth's own natural ability to repair itself built on the basis of our understating of its innate biological properties. Subsequently, it is essential for continued and genuine clinical and basic science research partnerships to enable challenging questions to be asked and answered as well as to enable the translation of key research findings. These studies will be inherently interdisciplinary at the interface between both biological and physical sciences and in particular relating to biomaterials, cell scaffolds, molecular biology, and stem cell biology.

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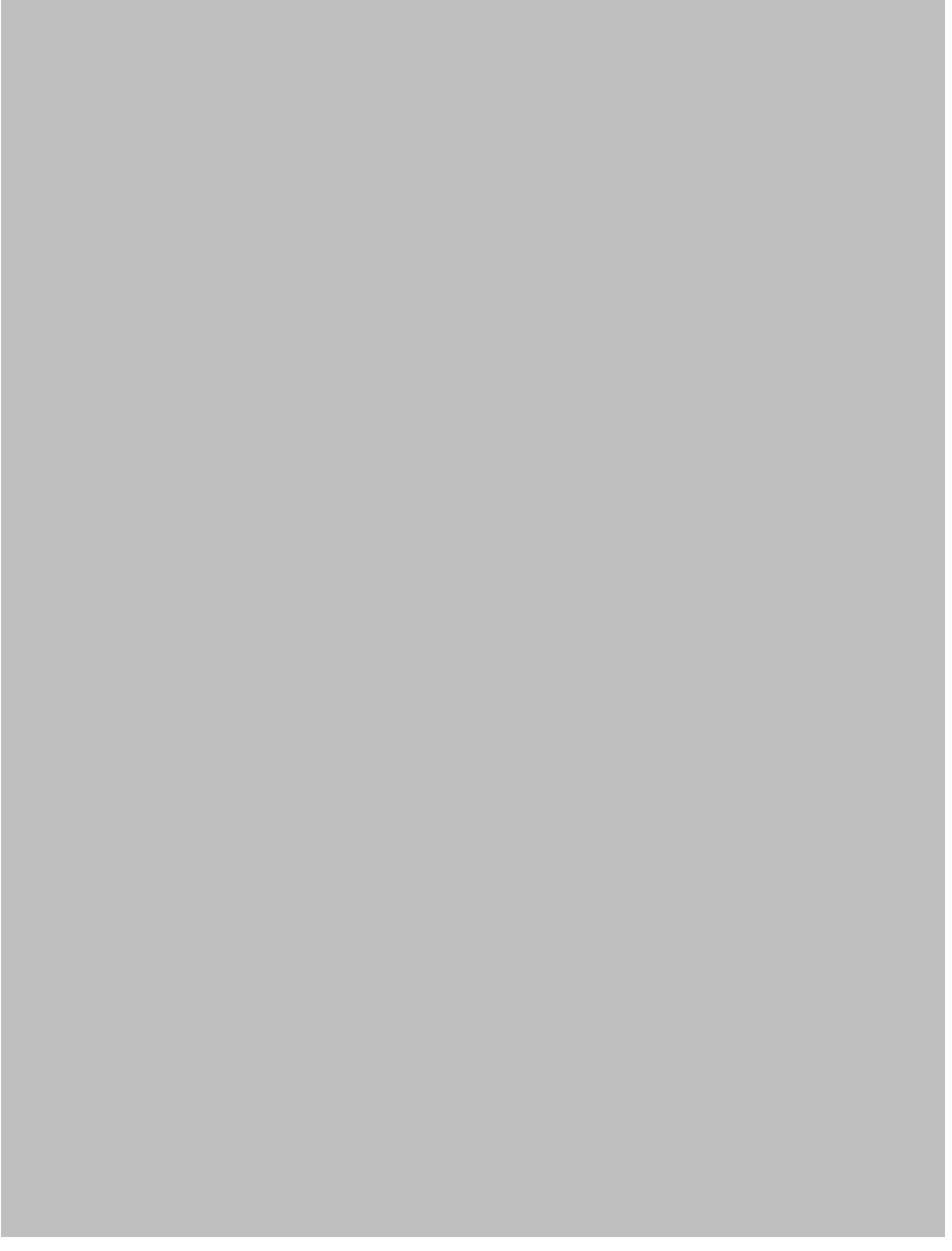
## CHAPTER 8

### PUBLICATION 7: ORIGINAL SCIENTIFIC MANUSCRIPT

Duncan HF, Smith AJ, Fleming GJP, Reid C, Smith G, Cooper PR. (2016). Release of bioactive dentine extracellular matrix components by histone deacetylase inhibitors (HDACi). *Int Endod J* doi: 10.1111/iej.12588. [Epub ahead of print]

It was hypothesised that HDACis could solubilise a range of DMCs from powdered dentine and owing to their acidic nature, would remove or alter the smear layer remaining after dentine preparation. The aim of this study was to compare the solubilisation of a range of DMCs by three HDACis with characterised extractants, prior to compositional analyses of the extracts using high-throughput antibody arrays and protein assays. The authors proposed that the experimentation would provide evidence that HDACis could extract potentially bioactive DMCs, which may work in synchrony with DPC-generated bioactive molecules to promote dentine-pulp repair processes. If successful, the process would highlight a further mechanism by which an HDACi containing dental restorative materials could promote regenerative events if placed clinically within vital pulp treatment.





the 1990s, the number of people in the UK who are aged 65 and over has increased from 10.5 million to 13.5 million, and the number of people aged 75 and over has increased from 4.5 million to 6.5 million (Office for National Statistics 2000).

There is a growing awareness of the need to address the needs of older people, and the need to ensure that they are able to live independently in their own homes for as long as possible. This has led to a number of initiatives, including the development of new housing schemes, the provision of services to support older people in their homes, and the development of new models of care.

One of the key challenges facing the housing sector is how to meet the needs of older people in a way that is sustainable and cost-effective. This requires a range of approaches, including the development of new housing schemes, the provision of services to support older people in their homes, and the development of new models of care.

The development of new housing schemes for older people is a complex task, and requires a range of skills and expertise. This includes the ability to understand the needs of older people, the ability to design and build housing that is suitable for older people, and the ability to manage the development process.

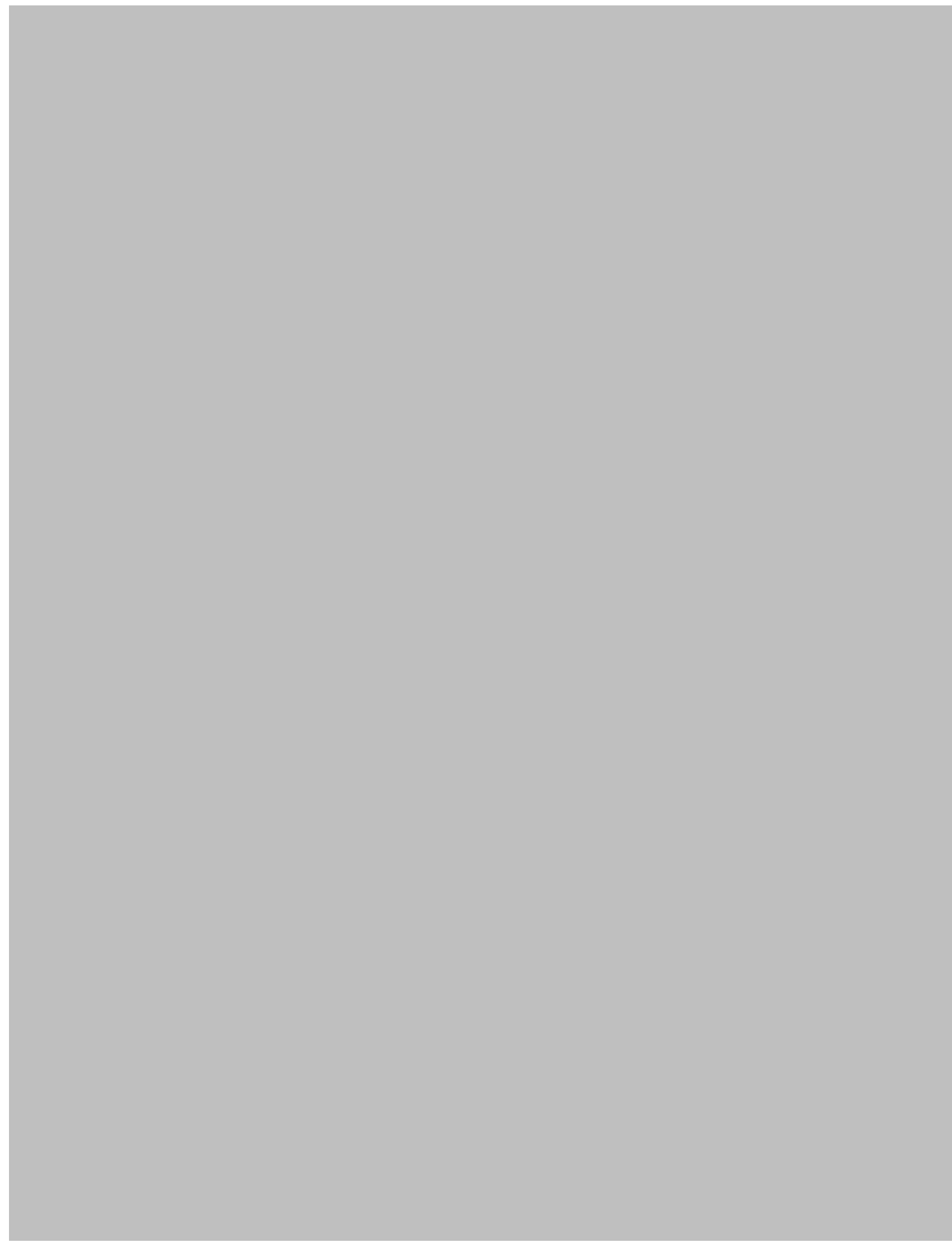
The provision of services to support older people in their homes is also a complex task, and requires a range of skills and expertise. This includes the ability to understand the needs of older people, the ability to design and deliver services that meet those needs, and the ability to manage the service.

The development of new models of care is also a complex task, and requires a range of skills and expertise. This includes the ability to understand the needs of older people, the ability to design and deliver new models of care, and the ability to manage the development process.

The housing sector is a key part of the social care system, and it is essential that it is able to meet the needs of older people in a way that is sustainable and cost-effective. This requires a range of approaches, including the development of new housing schemes, the provision of services to support older people in their homes, and the development of new models of care.

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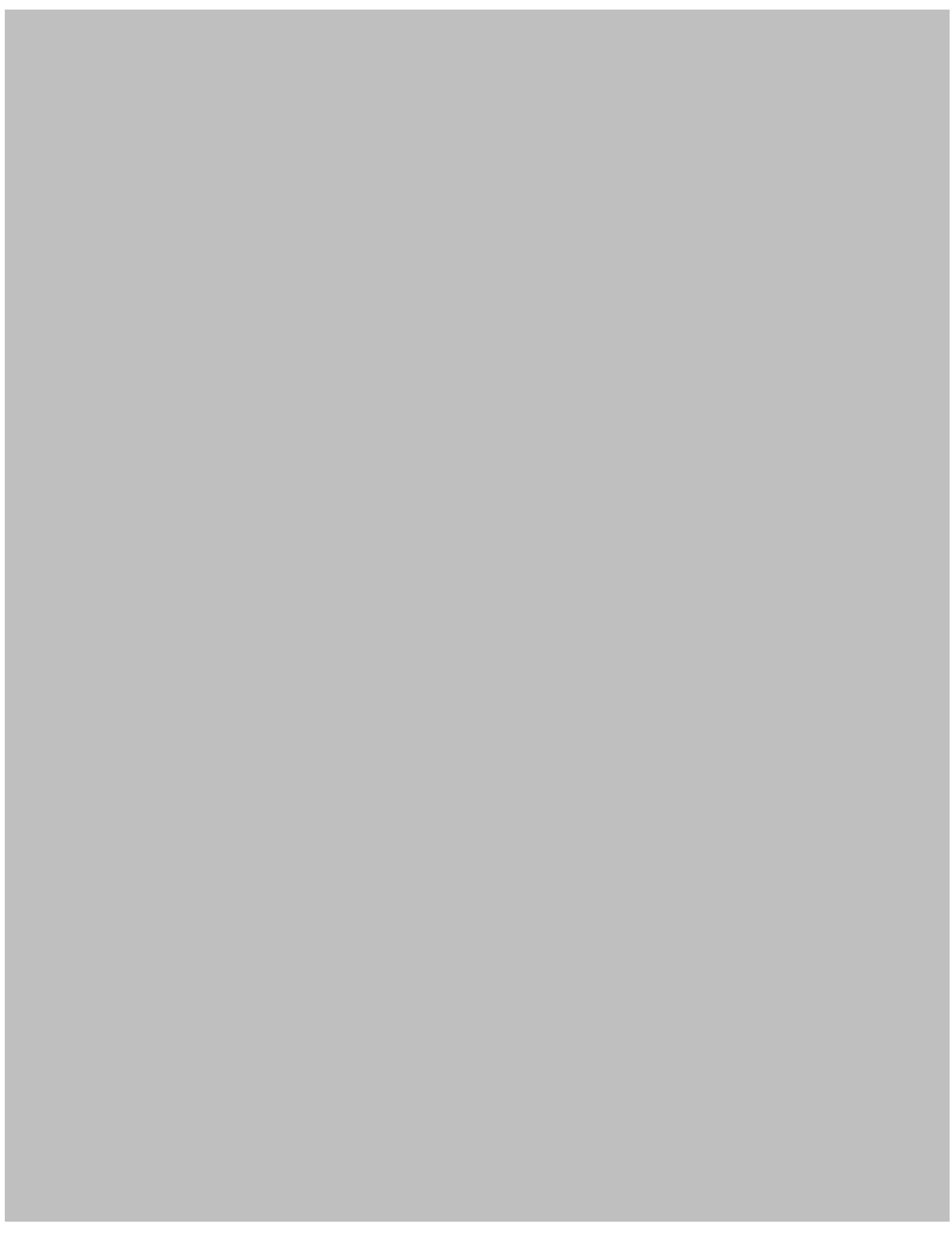


The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every entry, no matter how small, should be recorded to ensure the integrity of the financial data. This includes not only sales and purchases but also expenses and income. The document provides a detailed explanation of how to categorize these transactions and how to use a double-entry system to ensure that the books balance. It also discusses the importance of regular reconciliations to catch any errors early on.

The second part of the document focuses on the practical aspects of bookkeeping. It provides a step-by-step guide to setting up a chart of accounts, which is a list of all the accounts used in the business. This chart is essential for organizing the data and for generating financial statements. The document also discusses the importance of using a consistent and clear coding system for each account to facilitate data entry and retrieval.

The third part of the document discusses the various financial statements that can be generated from the bookkeeping records. These include the balance sheet, the income statement, and the cash flow statement. Each of these statements provides a different perspective on the financial health of the business. The document explains how to interpret these statements and how to use them to make informed decisions about the business's future.

The final part of the document discusses the importance of maintaining accurate records for tax purposes. It provides a detailed explanation of how to track deductible expenses and how to calculate taxable income. It also discusses the importance of keeping records for a sufficient period of time to support any claims made on a tax return.



The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every sale, purchase, and payment must be properly documented to ensure the integrity of the financial statements. This includes keeping receipts, invoices, and bank statements in a secure and organized manner.

Next, the document outlines the various methods used to collect and analyze financial data. It describes how different types of data, such as sales figures, expenses, and assets, are gathered and then processed to identify trends and patterns. This analysis is crucial for understanding the overall financial health of the organization and for making informed decisions about future operations.

The document also addresses the challenges of financial reporting, particularly in terms of ensuring accuracy and transparency. It highlights the need for strict adherence to accounting standards and the importance of regular audits to verify the reliability of the data. Additionally, it discusses the role of technology in streamlining financial processes and reducing the risk of human error.

Finally, the document concludes by emphasizing the long-term benefits of a robust financial reporting system. It notes that consistent and accurate reporting not only helps in identifying areas for improvement but also builds trust with stakeholders, including investors, creditors, and regulatory bodies. By maintaining high standards of financial integrity, organizations can ensure their long-term success and sustainability.

The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that proper record-keeping is essential for the success of any business and for the protection of the interests of all parties involved. The document then goes on to describe the various methods and techniques used to collect and analyze data, highlighting the importance of using reliable and valid sources of information. It also discusses the importance of using appropriate statistical methods to analyze the data and to draw meaningful conclusions from it. The document concludes by emphasizing the importance of using the results of the analysis to make informed decisions and to improve the performance of the business.



The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every entry, no matter how small, should be recorded to ensure the integrity of the financial data. This includes not only sales and purchases but also expenses and income. The document provides a detailed list of items that should be tracked, such as inventory levels, supplier payments, and customer orders. It also outlines the procedures for reconciling accounts and identifying discrepancies. The second part of the document focuses on the analysis of the recorded data. It describes various methods for interpreting the information, such as comparing current performance with historical trends and industry benchmarks. The document also discusses the implications of the data for decision-making, highlighting the need for timely and accurate reporting. Finally, the document concludes with a summary of the key findings and recommendations for future actions. It stresses the importance of continuous monitoring and adjustment to ensure the organization remains on track with its financial goals.

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The second section focuses on the role of the accounting system in providing a clear and concise overview of the company's financial health. It describes how a well-designed system can automate many of the routine tasks, such as invoicing and payroll processing, which saves time and reduces the risk of human error. The text highlights that a robust accounting system should be able to generate various reports, such as balance sheets, income statements, and cash flow statements, which are crucial for decision-making by management and investors.

In the third part, the document addresses the challenges of integrating different departments and systems. It points out that as a company grows, the complexity of its operations increases, and it becomes more difficult to ensure that all departments are using the same data and processes. The text suggests that implementing a unified accounting system can help in achieving this goal by providing a single source of truth for all financial data. It also mentions that regular communication and training are key to ensuring that all employees understand the importance of accurate record-keeping and how to use the accounting system effectively.

The final section discusses the future of accounting and the impact of technology. It notes that while traditional accounting methods have been around for centuries, the rise of digital technology has brought about significant changes. The text mentions that cloud-based accounting systems are becoming increasingly popular due to their flexibility and scalability. It also touches upon the use of artificial intelligence and machine learning in automating complex tasks and providing predictive analytics. The document concludes by stating that while technology is advancing rapidly, the fundamental principles of accounting remain the same, and it is the responsibility of accountants to stay updated and adapt to these changes.



**CHAPTER 9**

**GENERAL DISCUSSION**

## 9.1 Principal findings and discussion

Untreated dental pulp inflammation, as a result of microbial infection, leads to necrosis, apical periodontitis and apical abscess formation (Cooper *et al.*, 2011). Consequently, there is a need to develop biologically-based treatment protocols that harness the regenerative capacity of the pulp and maintain pulp vitality or replace irreversibly damaged or necrotic pulp tissue (Ferracane *et al.*, 2010; Smith *et al.*, 2016). Although the potential regenerative benefits of epigenetic-based therapeutic intervention have recently been highlighted in periodontology (Barros & Offenbacher, 2014), within endodontology there have been no previous investigations. Therefore, the main focus of the current research project was to investigate a novel role for the epigenetic-modifying agents, HDACis, in augmenting regenerative processes within vital pulp treatment.

Within this research project, the hypothesis that HDACis could positively affect cell proliferation and differentiation to accelerate mineralisation events in dental pulp-derived cells was supported. Two broad spectrum HDACis (TSA and VPA) added to a dental-papillae derived cell line (MDPC-23), reduced proliferation and increased mineralisation dose-dependently, which had not previously been reported. In addition, the application of HDACi, in relatively low concentrations (12.5-400 nM TSA; 0.125-5 mM VPA), adversely influenced cell viability and cell cycle only at the highest concentrations employed (400 nM TSA; 5 mM VPA) (Chapter 4; Duncan *et al.*, 2012). Although this work demonstrated the principle of HDACi-induced pulp cell mineralisation, it also generated further questions regarding the potential nature of the response in primary/non-transformed cell cultures, which have been demonstrated to react differently to HDACi in other cellular systems (Ungerstedt *et al.*, 2005; Lee *et al.*, 2010). Subsequent primary DPC experimentation supported the hypothesis,

that HDACi-induced reparative-related cellular responses at concentrations, which did not stimulate significant cytotoxic effects. Comparison between the two studies, however, revealed marked differences between the primary DPC and immortalised cell-line responses. Notably, the same HDACis (TSA and VPA) induced mineralisation at concentrations 10-fold lower in primary DPCs compared with the pulp cell-line. In terms of HDACi dosage, an increase in mineralisation per cell was evident after 24 h application of HDACi in the primary DPC group, but not after constant HDACi dosage up to 14 days, which conversely resulted in an inhibition of cell differentiation. This effect was not evident in the MDPC-23 cell cultures in which constant exposure to HDACi increased mineralisation. Primary DPC experimentation revealed no loss of cell viability or increase in apoptosis at the highest concentrations applied (400 nM TSA; 5 mM VPA), indicative a resistance of DPCs to HDACi-mediated toxicity not evident in MDPC-23 cells (Chapter 5; Duncan *et al.*, 2013). From a translational perspective, the stimulation of mineralisation in DPCs without evidence of cell toxicity after a 24 h exposure to HDACi, supports the potential chairside application of HDACis as pulp capping agents within clinical vital pulp treatment protocols.

The conclusion that 24 h HDACi application would induce reparative responses in primary DPCs required further investigation. As a result, the molecular mechanisms driving the regenerative properties of a clinically-approved HDACi, SAHA (Grant *et al.*, 2007) were investigated. The studies focused on the expression of novel transcripts and the upregulation of mineralisation-associated pathways at the 24 h and 14 day time-points. The hypothesis that the mechanism behind the regenerative properties of SAHA, related to the transcriptomic changes which modulate selected mineralisation-associated pathways and that certain novel markers interact with SAHA to promote and augment pulpal regenerative

processes was supported. The expression of several unreported and previously characterised genes, associated with mineralisation processes, were upregulated at both 24 h and 14 days. After gene expression pathway analysis, the significant upregulation of several members of the MMP family of endopeptidases (MMP-9, MMP-13 and MMP-17) and endochondral ossification pathway were highlighted as a potential mechanism for SAHA-stimulated mineralisation in DPCs (Duncan *et al.*, 2015). Although the interaction between HDACi and MMPs was highlighted previously for transformed (Lin *et al.*, 2012) and primary cell populations (Mayo *et al.*, 2003; Schroeder *et al.*, 2007), there had been no previous attempts to link this interaction to reparative processes in the dental pulp or elsewhere. Furthermore, experimentation which specifically investigated the interaction of HDACi and MMP-13 demonstrated that SAHA promoted DPC mineralisation and induced MMP-13 expression and activity at both 24 h and 14 days. Interestingly, an increase in MMP-13 also mediated a SAHA-induced increase in DPC migration (Chapter 6; Duncan *et al.*, 2016a).

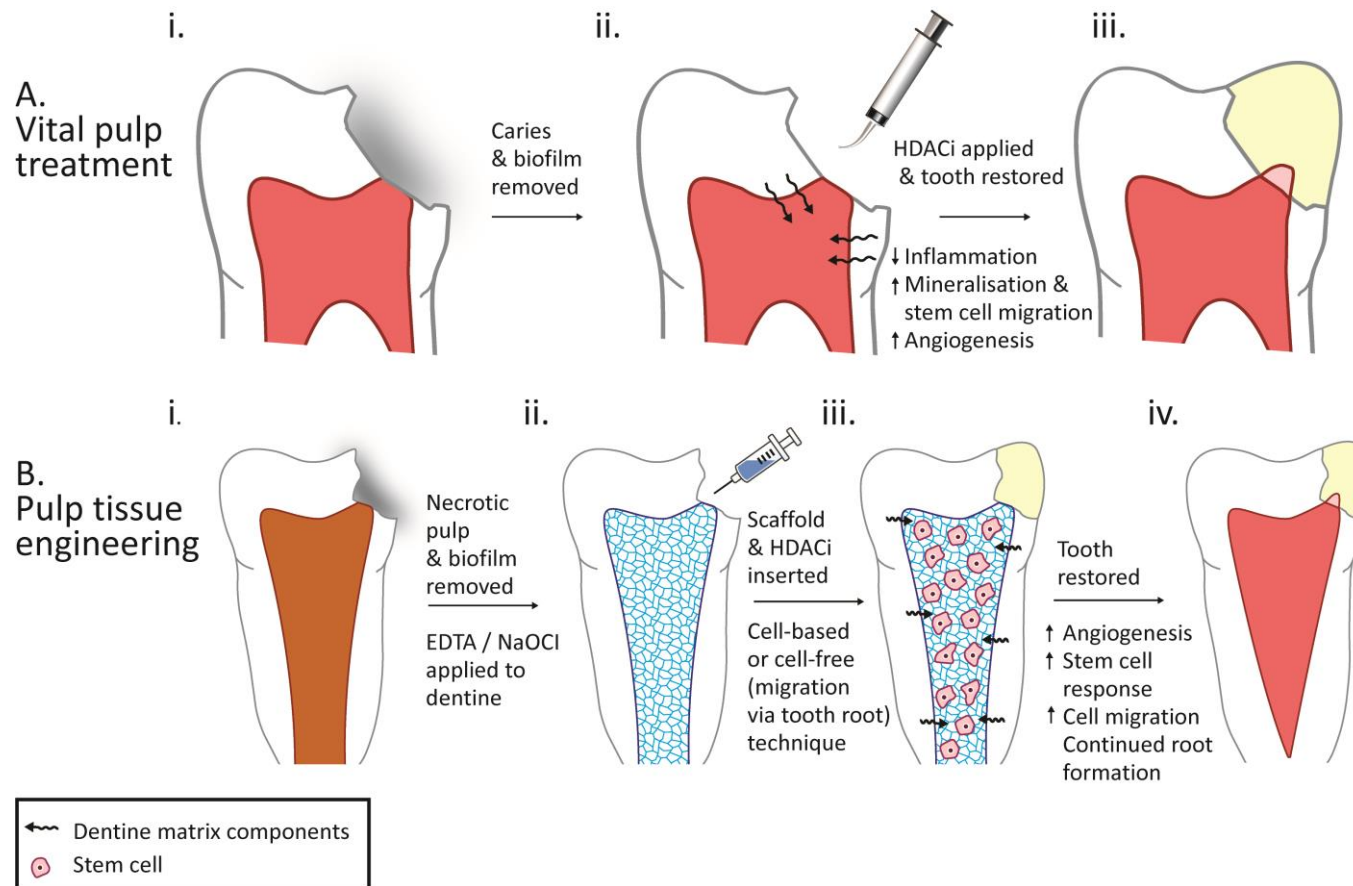
The hypothesis that HDACis would remove or alter the smear layer remaining after dentine preparation was not supported as all three HDACis studied (TSA, VPA and SAHA) did not remove or alter the smear layer. However, the hypothesis that HDACis would solubilise a range of DMCs was supported as TSA, VPA and SAHA were able to solubilise a range of DMCs which may promote bioactive repair responses clinically. Dentine is known to be a rich source of bioactive molecules and the HDACi-DMC extracts contained a range of GFs previously identified as being released from dentine by endodontic irrigants (Finkelman *et al.*, 1990; Roberts-Clark & Smith, 2000) and the dental materials Ca(OH)<sub>2</sub> and MTA (Graham *et al.*, 2006; Tomson *et al.*, 2007), as well as other uncharacterised GFs such as GDNF and BDNF (Chapter 8; Duncan *et al.*, 2016b). Importantly, the HDACis extracted a range of GFs

from dentine less efficiently than the well-characterised extractant EDTA (TGF- $\beta$ -1, PDGF-AA and VEGF-A), but more effectively for others (GDF-15, IGF-1, EGRF-1, NGFR, SCF-R), while notably each HDACi exhibited a different extraction profile. The observations that HDACi solubilised DMCs have clinical relevance as both pulp capping agents and pulp engineering scaffolds contact dentine at the periphery of the pulp exposure or in the root canal system (Fig. 9.1), thereafter releasing bioactive DMCs, which promote reparative events (Graham *et al.*, 2006). The project established a novel potential additional mechanism for HDACi action on reparative and regenerative dentine-pulp events in addition to direct cellular epigenetic actions, which have not previously been reported in the dental literature.

## 9.2 Future work

The present work offers detailed *in vitro* analyses of HDACi-induced DPC repair processes, highlighting novel transcripts and molecular pathways stimulated by HDACi, as well as characterising the release of DMCs by these inhibitors. A previously unreported role for epigenetic modification in the pulpal wound healing response was demonstrated, which provides a potential opportunity for therapeutic HDACi intervention during regenerative endodontic procedures (Fig. 9.1). In order to progress the translational potential of the study, the suggestion that the clinical topical application of HDACi promotes reparative processes during vital pulp or pulp engineering procedures (Duncan *et al.*, 2016c) requires further investigation. An *in vivo* assessment, using animal models, of the healthy and inflamed pulpal responses to HDAC and MMP-13 inhibitors would be necessary to determine the clinical potential of the current *in vitro* studies. This work also provides a basis for further





**Figure 9.1 Schematic diagram highlighting the therapeutic potential of HDACi in regenerative endodontics. A. Vital pulp treatment. i.** Deep carious lesion exposes pulp tissue. **ii.** A single dose of HDACi topically applied to exposed pulp, potentially as a component of a dental restorative material [mineral trioxide aggregate (MTA), calcium hydroxide, resin-based composite (RBC)] promotes tissue-repair processes, including increased mineralisation, modulated inflammation, organisation of mineralisation processes and cell migration. **iii.** Tooth suitably restored over capping material with amalgam, RBC or glass ionomer and mineralised bridge formation evident under dental pulp capping material. **B. Pulp tissue engineering. i.** Pulp necrosis caused by trauma, caries or microleakage around restoration. **ii.** Necrotic tooth chemo-mechanical debrided and EDTA, NaOCl applied to dentine to release matrix components (Conde *et al.*, 2015). **iii.** HDACi applied within a cell or non-cell based scaffold stimulates further growth factor release, migration of cells and differentiation processes. **iv.** Tooth restored and new pulp-like tissue formed promoting continued root growth and restoring tooth tissue vitality.

investigations into several related areas of research which are described below, in the immediate-, medium- and long-term, which should increase our understanding of the influence of epigenetics and specifically HDACi on dentine-pulp repair and regeneration.

The experimental work presented has currently focussed on the actions of three HDACis (TSA, VPA and SAHA), which offer pan-specific inhibition for zinc-dependent HDACs being effective against all Class I (HDAC-1, -2, -3, -8), Class II (HDAC-4, -5, -6, -9, -10) and Class IV (HDAC-11) enzymes (Marks & Dokmanovic, 2005), but not the Class III HDACs known as sirtuins (Haigis & Guarente, 2006). As there are multiple, opposing cellular effects of HDAC (Balasubramanian *et al.*, 2009) and the expression of class II HDAC enzymes is tissue dependent (Verdin *et al.*, 2003), the development of isoform specific HDACi has been advocated (Khan *et al.*, 2008; Muraglia *et al.*, 2008). Therefore, in the immediate-term I would aim to focus the research in two specific areas; (i) expression analyses of individual class II HDAC enzymes in pulp tissue and changes in expression during disease and repair, with a view to investigating isoform-specific HDACi; and (ii) the effects of HDACi and MMP-13i in vital pulp treatment *in vivo*, initially in an animal model and later using HDAC-specific knock-out mice (Lee & Partridge, 2010; Kim *et al.*, 2012).

Depending on the results of the next phase of experimentation, medium-term research plans could include an investigation of a potential anti-inflammatory HDACi role in pulp cell cultures and *in vivo* models of pulpal inflammation (Renard *et al.*, 2016) with optimisation of the dental restorative material aspects for the delivery of HDACi to the pulpal wound site (Paranjpe *et al.*, 2008). Long-term research goals would be to engage in a prospective controlled clinical trial investigating the histological and clinical efficacy of HDACi as an adjunct to vital pulp treatment protocols (Nair *et al.*, 2008) or as a scaffold morphogen

to functionally assist pulp tissue engineering procedures (Rosa *et al.*, 2013; Albuquerque *et al.*, 2014; Piva *et al.*, 2014; Conde *et al.*, 2015) (Fig. 9.1). Further epigenetic-modifier based projects can be developed alongside the proposed studies since although the current project has concentrated on the effects of one group of epigenetic-modifying agents, other agents targeting DNA and histone methylation also provide therapeutic candidates for treatment of damaged pulp tissue (Zhang *et al.*, 2015). The use of DNA methyl transferase inhibitors alone or in combination with HDACis presents a potentially exciting area of future research (Mahpatra *et al.*, 2010).

### **9.3 Conclusions**

Concerns over the destructive nature of dental treatment protocols have led the dental profession to examine methodologies that develop regenerative treatment protocols and promote minimally-invasive, biologically-based dental restorative solutions (Banerjee, 2013; Schmalz & Smith, 2014). Within endodontics, this has resulted in a shift towards regenerative endodontic procedures aimed to protect the pulp and harness its natural regenerative capacity or facilitate the engineering of new pulp tissue (Cordeiro *et al.*, 2008) when irreversibly inflamed or necrotic. Traditionally, vital pulp treatments have unpredictable clinical outcomes, in part due to poor inflammatory control or low quality mineralised tissue formation at the exposure site (Cox *et al.*, 1996; Nair *et al.*, 2008). The research presented highlights an exciting opportunity for the topical use of the epigenetic-modifying-agents, HDACis, in augmenting tissue repair processes during regenerative endodontic procedures. Data presented have demonstrated the previously unreported influence of epigenetics and in particular HDAC function on the molecular signalling

controlling cell fate, cell behaviour and cell differentiation, although, the complexity of epigenetic modifications was also highlighted. The paucity of published laboratory data necessitates future research prior to, and alongside, any proposed changes to clinical treatment protocols. The observation that HDACi promoted both DPC mineralisation and migration modulated by changes in MMP activity, highlighted other potential therapeutic targets or combinatorial therapies that could provide a focus for future research and clinical application in this area. Translationally, HDACi-induced DMC release presents further evidence for a potential benefit of augmenting a dental restorative material or tissue engineering scaffolds with HDACi as a basis for new biologically-based endodontic treatment protocols in the future.

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## **Appendix 1**





## Data in Brief

# Transcriptional profiling of suberoylanilide hydroxamic acid (SAHA) regulated genes in mineralizing dental pulp cells at early and late time points



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## ABSTRACT

Dental pulp tissue can be damaged by a range of irritants, however, if the irritation is removed and/or the tooth is adequately restored, pulp regeneration is possible (Mjör and Tronstad, 1974 [1]). At present, dental restorative materials limit healing by impairing mineralization and repair processes and as a result new biologically-based materials are being developed (Ferracane et al., 2010 [2]). Previous studies have highlighted the benefit of epigenetic modification by histone deacetylase inhibitor (HDACi) application to dental pulp cells (DPCs), which induces changes to chromatin architecture, promoting gene expression and cellular-reparative events (Duncan et al., 2013 [3]; Paino et al., 2014 [4]). In this study a genome-wide transcription profiling in epigenetically-modified mineralizing primary DPC cultures was performed, at relatively early and late time-points, to identify differentially regulated transcripts that may provide novel therapeutic targets for use in restorative dentistry. Here we provide detailed methods and analysis on these microarray data which has been deposited in Gene Expression Omnibus (GEO): GSE67175.

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## Specifications

Organism/cell line/tissue	<i>Rattus norvegicus</i> /dental pulp tissue
Sex	Male
Sequencer or array type	Agilent Sure Print 4x44K array in situ oligonucleotide (G2519F)
Data format	Raw and processed
Experimental factors	Cultured primary dental pulp cells (DPCs) extirpated from the incisor teeth of Wistar Hannover rats aged 25–30 days
Experimental features	High-throughput investigation of genome-wide transcripts, epigenetically regulated by histone deacetylase inhibitors (HDACi). Four independent biological tissue samples were cultured at two different time points (24 h and 14 days) under mineralizing conditions in the presence or absence of the HDACi, SAHA.
Consent	N/A
Sample source location	University of Dublin, Dublin, Ireland, Europe

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## 1. Direct link to deposited data

<http://www.ncbi.nlm.nih.gov/gds/?term=GSE67175>.

## 2. Experimental design, materials and methods

## 2.1. Primary cell culture

Primary DPCs were isolated from the extirpated pulp tissue of freshly extracted rodent incisor teeth using enzymatic disaggregation procedure as previously described [5]. The maxillary and mandibular incisor teeth were dissected from male Wistar Hannover rats aged 25–30 days and weighing 120–140 g. The pulp tissue was extirpated from the pulp chamber, minced with a scalpel into 1 mm<sup>3</sup> pieces and transferred into Hank's balanced salt solution (Sigma-Aldrich, Arklow, Ireland) prior to incubation at 37 °C, 5% CO<sub>2</sub> for 30 min (MCO-18AC incubator, Sanyo Electric, Osaka, Japan). Cells were transferred to an equal volume of supplemented α-MEM (Biosera, East Sussex, UK) containing 1% (w/v) penicillin/streptomycin (Sigma-Aldrich) and 10% (v/v) fetal calf serum (FCS) (Biosera). A single cell suspension was

obtained by passing through a 70  $\mu\text{m}$  cell sieve (BD Biosciences, Oxford, UK) prior to centrifugation and re-suspension in 1 ml supplemented  $\alpha$ -MEM. DPCs were expanded under standard culture conditions to passage 2. The DPCs were seeded in supplemented  $\alpha$ -MEM for 3 days (experimental day 0), and then either cultured for 24 h in supplemented mineralizing medium containing 1  $\mu\text{M}$  SAHA prior to harvest (24 h samples) or incubated with an HDACi-free mineralizing medium for a further 13 days (14 day samples). Control samples contained cells cultured in mineralizing medium without SAHA.

## 2.2. Suberoylanilide hydroxamic acid (SAHA) preparation

A 5 mM stock solution of SAHA (*N*-hydroxy-*N'*-phenyl-octanediamide) (Sigma-Aldrich), in dimethyl sulfoxide (DMSO) was diluted in phosphate buffered saline (PBS) (Sigma-Aldrich) prior to further dilution to 1  $\mu\text{M}$  with supplemented  $\alpha$ -MEM.

## 2.3. RNA isolation, cDNA and labeled cRNA preparation

Cultures were detached (trypsin/EDTA), homogenized and RNA extracted using the RNeasy mini kit (Qiagen, West Sussex, UK), before being quantified spectrophotometrically (Nanodrop 2000, Thermo Fisher Scientific). A 75 ng aliquot of total RNA was converted to cDNA using an oligo dT-promoter primer and Affinity-Script-RT (Agilent Technologies), prior to being labeled with either Cyanine 3-CTP or Cyanine 5-CTP using the Two-Color Low Input Quick Amp labeling Kit (Agilent Technologies, Cork, Ireland) and transcribed to cRNA (Agilent Technologies). The cRNA was purified using the RNeasy mini kit (Qiagen) and Cy3, Cy5 concentration, RNA absorbance 260/280 nm and cRNA concentrations determined spectrophotometrically (Nanodrop 2000, Wilmington, DE, USA). This enabled specific activity and target yields to be calculated prior to microarray experimentation.

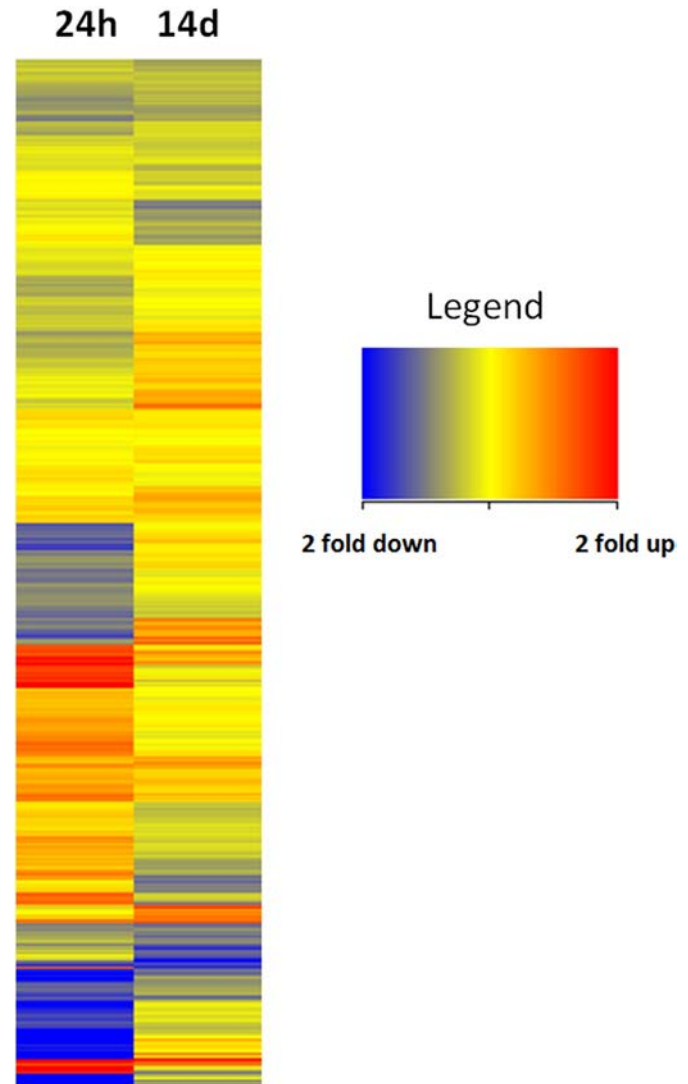
## 2.4. Microarray experimentation and analysis

The Agilent 4x44K v3 whole rat genome oligonucleotide gene expression microarray (Agilent Technologies) was used to analyze the transcript profiles of HDACi treated (1  $\mu\text{M}$  SAHA) and untreated DPC cultures at both 24 h and 14 days under mineralizing conditions. The microarray analyses were performed on quadruplicate independent DPC cultures ( $n = 4$ ) at both time-points. A total of 825 ng of labeled cRNA from each sample (treated and untreated) was loaded onto an individual array according to the manufacturer's instructions and co-hybridized at 65  $^{\circ}\text{C}$  for 17 h, washed and scanned in GenePix-Personal 4100A, Pro 6.1 (Axon, Molecular Devices, CA, USA) at a resolution of 5  $\mu\text{m}$ .

Raw data were exported to GeneSpring GX12 and signals for each replicate spot were background corrected and normalized using Lowess transformation. Log<sub>2</sub> fluorescent intensity ratios were generated for each replicate spot and averaged. Genes that were differentially expressed (>2.0 fold) in the SAHA group relative to control were identified after passing a t-test ( $p < 0.05$ ), post-hoc test (Storey with bootstrapping) with a corrected q value of 0.05 (Fig. 1). Genes in the expression datasets were first 'ranked' based on Log<sub>2</sub> values from the highest to lowest for both groups at both time points, prior to hierarchical clustering being used to group gene expression in each condition using the default settings in Genespring GX12. Gene Ontology (GO) was evaluated using Go-Elite ([http://www.genmapp.org/go\\_elite](http://www.genmapp.org/go_elite)) [6], which is designed to identify a minimal non-redundant set of biological Ontology terms to describe a particular set of genes (Fig. 2).

## 2.5. Validation of microarray data by quantitative RT-PCR analysis

In order to validate the microarray, isolated RNA was converted to single-stranded cDNA using the TaqMan™ reverse transcriptase kit and 50  $\mu\text{M}$  random hexamers (Life Technologies) and the

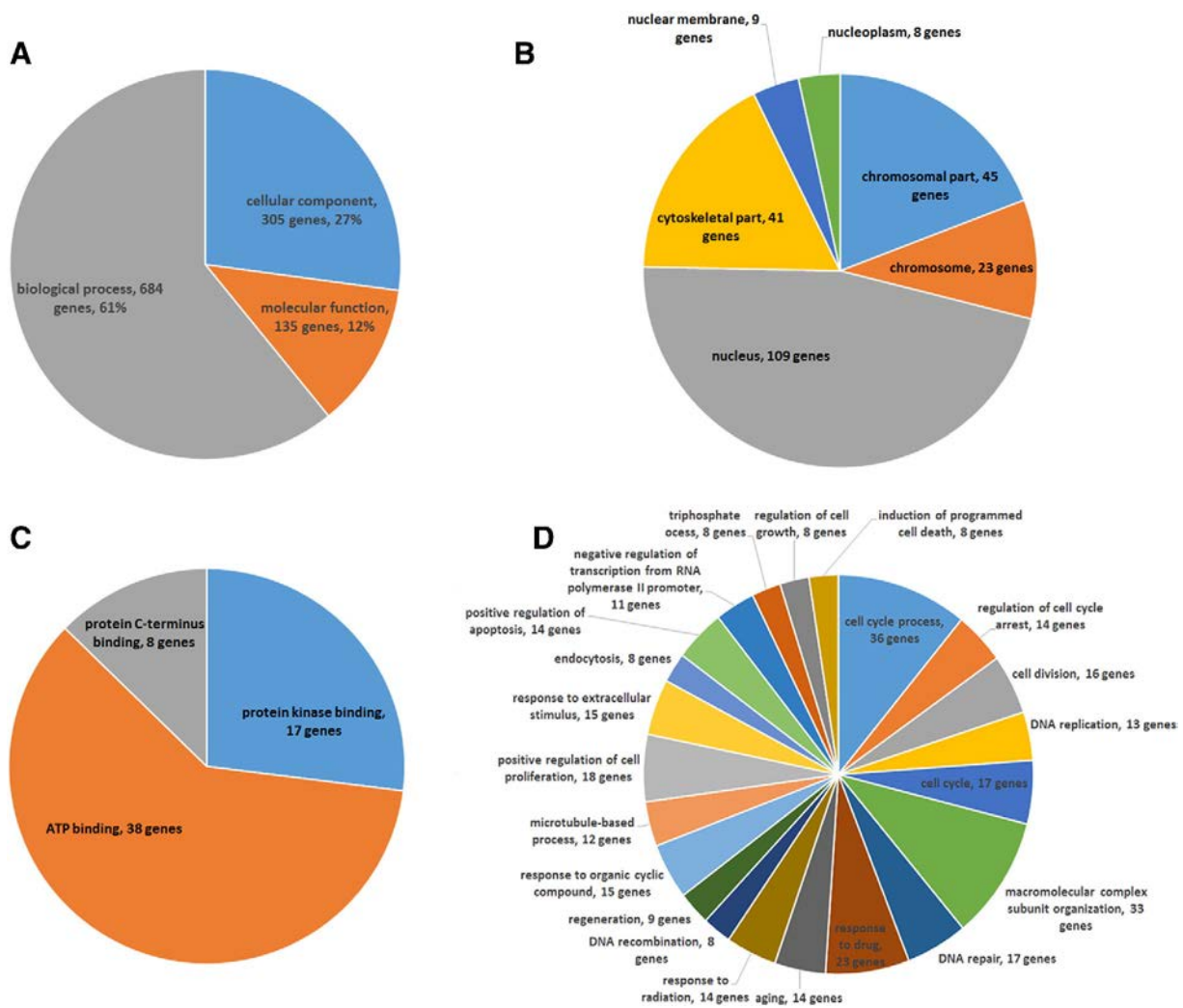


**Fig. 1.** A comparison of >2 fold gene expression at 24 h and 14 days in DPCs after exposure to SAHA for 24 h. The 'heat-map' shows expression patterns for all present genes.

concentrations determined spectrophotometrically (Nanodrop 2000, Thermo Fisher Scientific). A range of transcripts was selected based on their levels of differential regulation. The q-RT-PCR analysis was performed for rat genes using specific primers (Invitrogen, Life Technologies, Thermo Scientific, Paisley, UK) for *Dab1*, *Gsta4*, *Hist1h1b*, *Ska1*, *Epha3*, *Krt18*, *Mal2* and *Rasgrp13*. Primers for each selected gene were designed using Primer3Plus tools (<http://primer3plus.com/>). Sequence identity of the amplicon was confirmed with NCBI Basic Local Alignment Search Tool (blast) software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Synthesized cDNA was amplified and PCRs performed using the Applied Biosystems 7500 Fast Real-Time PCR thermal cycler (Applied Biosystems), being subjected to a designated number of amplification cycles (40 cycles), where a typical cycle was 95  $^{\circ}\text{C}$  for 3 s and 60  $^{\circ}\text{C}$  for 30 s. Real-time PCR data were normalized to  $\beta$ -actin. The upregulated and downregulated transcripts assayed by RT = PCR were consistent with the results obtained from DNA microarray (Table 1).

## 3. Discussion

In this report we describe detailed technical methods to reproduce the high-throughput gene analysis using the whole rat genome oligo microarray kit (Agilent, G2519F, Ireland) for mineralizing primary DPC



**Fig. 2.** Functional categories of the genes showing significant >2 fold expression change in DPCs at 24 h relative to control. A, Biological categories of the transcripts were assigned with GoElite ([http://www.genmapp.org/go\\_elite](http://www.genmapp.org/go_elite)) analysis software and further subdivided by category; B, Cellular component; C, molecular function; and D, biological process. Only GoTerms categories with greater than 7 transcript members are included.

**Table 1**

A comparison of microarray and quantitative RT-PCR for selected genes at 24 h and 14 days.

Gene	Fold change cDNA microarray (24 h)	Fold change RT-PCR (24 h)
Dab1	4.13	5.17
Gsta4	8.21	14.5
Hist1h1b	-7.83	-13.0
Ska1	-97.2	-72.5
Gene	Fold change cDNA microarray (14 days)	Fold change RT-PCR (14 days)
Epha3	-2.4	-2.06
Krt18	-2.8	-2.65
Mal2	2.83	2.73
Rasgrp13	2.33	2.32

cultures in the presence and absence of a HDACi. The validated gene expression data demonstrates the relevant expression profile signature of differentially expressed genes during mineralization and identifies several transcripts not previously associated with this process. This data can contribute to future investigations examining transcriptional changes that promote tissue repair processes in the dental pulp.

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**Further Reading**

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## **Appendix 2**

## Publications, Presentations and Awards

### Research manuscripts from this doctorate published in peer-reviewed journals:

- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2012). Histone deacetylase inhibitors induced differentiation and accelerated mineralization of pulp-derived cells. *J Endod* 38:339-345. doi: 10.1016/j.joen.2011.12.014.  
PMID: 22341071
- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2013). HDACi epigenetically promote reparative events in primary dental pulp cells. *Exp Cell Res* 319:1534-1543. doi: 10.1016/j.yexcr.2013.02.022.  
PMID: 23562654
- **Duncan HF**, Smith AJ, Fleming GJP, Moran GP, Cooper PR (2015). Transcriptional profiling of suberoylanilide hydroxamic acid (SAHA) regulated genes in mineralizing dental pulp cells at early and late time points. *Genom Data* 5:391-393. doi: 10.1016/j.gdata.2015.07.013.  
PMID: 26484294
- **Duncan HF**, Smith AJ, Fleming GJP, Partridge NC, Shimizu E, Moran GP, Cooper PR (2016). The histone-deacetylase-inhibitor suberoylanilide hydroxamic acid promotes dental pulp repair mechanisms through modulation of matrix metalloproteinase-13 activity. *J Cell Physiol* 231:798-816. doi: 10.1002/jcp.25128.  
PMID: 26264761
- **Duncan HF**, Smith AJ, Fleming GP, Reid C, Smith G, Cooper PR (2016). Release of bio-active dentine extracellular matrix components by histone deacetylase inhibitors (HDACi) *Int Endod J*. doi: 10.1111/iej.12588. [Epub ahead of print]  
PMID: 26609946

### Review manuscripts from this doctorate published in peer-reviewed journals:

- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2011). HDACi: cellular effects, opportunities for restorative dentistry. *J Dent Res* 90:1377-1388. doi: 10.1177/0022034511406919.  
PMID: 21536971
- **Duncan HF**, Smith AJ, Fleming GP, Cooper PR (2016). Epigenetic modulation of dental pulp stem cells: implications for regenerative endodontics. *Int Endod J*. doi: 10.1111/iej.12475. [Epub ahead of print]  
PMID: 26011759
- Smith AJ, **Duncan HF**, Diogenes A, Simon S, Cooper PR (2016). Exploiting the bioactive properties of dentin-pulp in regenerative endodontics. *J Endod* 42:47-56. doi.org/10.1016/j.joen.2015.10.019.  
PMID: 26699924

**Published book chapters related to this doctorate:**

- **Duncan HF** (2011). Pulp capping in a mature tooth. In: Pitt Ford's Problem-Based Learning in Endodontology. (Editors, Patel S & Duncan HF), Chapter 3.3, pp. 75-83. 1<sup>st</sup> ed., Blackwell Publishing, Wiley Blackwell, Oxford, UK.
- **Duncan HF**, Cooper PR (2016). Epigenetics of dental stem cells. In: Dental Stem Cells: Regenerative Potential. (Editors, Zavan B & Bressan E), Chapter 5, pp. 73-84, 1<sup>st</sup> ed., Springer International Publishing AG, Cham, Switzerland.
- **Duncan HF**, Smith AJ, Cooper PR (2016). Maintaining dental pulp vitality. In: Harty's Endodontics in Clinical Practice. (Editor, Chong BS), Chapter 5, pp. 55-69, 7<sup>th</sup> ed., Churchill Livingstone, Elsevier, Edinburgh, UK.

**Published peer-reviewed conference abstracts from this doctorate:**

- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2012). Histone-deacetylase-inhibitors promote tissue-related repair mechanisms in primary dental pulp cells. PER IADR Helsinki, Finland, September 2012.
- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2013). Histone-deacetylase-inhibitors epigenetically promote reparative events in primary dental pulp. IADR General Session, Seattle, USA, March 2013.
- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2014). The effects of the histone-deacetylase-inhibitor suberoylanilide hydroxamic acid on pulp cells. PER IADR, Dubrovnik, Croatia, September 2014.
- **Duncan HF**, Smith AJ, Fleming GJP, Partridge NC, Shimizu E, Moran GP, Cooper PR (2015). Histone-deacetylase-inhibition promotes pulpal repair mechanisms partly by modulating matrix-metalloproteinase-13 activity. IADR General Session, Boston, USA, March 2015.

**Peer-reviewed conference abstracts (without published abstracts) from this doctorate:**

- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2011). Histone deacetylase inhibitors induced differentiation and accelerated mineralization of pulp-derived cells. Irish Division of the IADR, Annual Scientific Meeting, Dublin, Ireland, October 2011.
- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2015). Epigenetic modulation of dental pulp stem cells using pharmacological inhibitors: Endodontic implications. European Society of Endodontology, Biennial Conference, Barcelona, Spain, September 2015.

#### **Invited oral conference presentations from this doctorate:**

- **Duncan HF** (2009). Vital pulp treatment: where, when and what to use? Irish Dental Association, Annual Scientific Meeting, Wexford, Ireland, October 2009.
- **Duncan HF** (2011). HDACi, epigenetics and new regenerative biomaterials. Irish Endodontic Society, Annual Scientific Meeting, Trinity College Dublin, Dublin, Ireland, March 2011.
- **Duncan HF** (2011). Vital pulp treatment: regenerative developments and clinical considerations. Dental Faculty, Royal College of Surgeons of Ireland, Annual Scientific Meeting, Dublin, Ireland, October 2011.
- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2013). Epigenetics and basic science research: clinical translation in vital pulp treatment. Odontological Society, Royal College of Physicians Ireland, Dublin, Ireland, February 2013.
- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2013). Promoting pulpal repair mechanisms using HDACi: clinical translations in vital pulp treatment. New York University, Annual Research Meeting, Department of Basic Science, New York University, New York, USA, August 2013.
- **Duncan HF**, Smith AJ, Fleming GJP, Cooper PR (2014). The effects of the histone-deacetylase-inhibitor suberoylanilide hydroxamic acid on pulp cells. Odontological Society, Royal College of Physicians Ireland, Dublin, Ireland, November 2014.
- **Duncan HF** (2015). Dental pulp; how do I diagnose pulp disease and how to treat with new technologies? Swiss Endodontic Society, Annual Scientific Meeting, Lucerne, Switzerland, January 2015.
- **Duncan HF** (2015). Vital pulp treatment: old problems, new epigenetic solutions. International College of Dentistry, European Conference, Royal College of Surgeons Ireland, Dublin, Ireland, October 2015.
- **Duncan HF** (2016). Pulp biology update: Is regeneration possible with new therapeutic strategies? Dutch Society of Endodontics, Annual Scientific Meeting, Amsterdam, the Netherlands, April 2016.

#### **Research prizes and grant awards:**

- **Irish Endodontic Society Research Grant.** Awarded May 2009, total €30,000.
- **Rodney Dockrell Bursary Fund.** Awarded November 2009, total €1,000.
- **Irish IADR Senior Postgraduate Prize.** Awarded PER IADR Helsinki, September 2012; Hatton entry IADR Seattle 2013.
- **Sir John Gray IADR Fellowship.** Awarded October 2012, total \$10,000. Three month and 6 week fellowship to Professor Nicola Partridge's laboratory, New York University 2013 and 2016.
- **European Society of Endodontology Annual Research Grant.** Awarded November 2012, total €10,000.