University of Technology, Sydney

The Characterisation of Adipose Derived Stem Cells on Coralline Scaffolds for Bone Tissue Engineering.

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Abbreviations

2-Dimenstional-Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis	2D-SDS-PAGE
Adipose Derived Stem Cells	ADSCs
3,3´-diaminobenzidine	DAB
Bovine Serum Albumin	BSA
Beta-tricalcium phosphate	β-TCP
Calcium Carbonate	CC
Coralline hydroxyapatite	cHA
Dulbecco's Modified Eagle's Medium	DMEM
Embryonic Stem Cells	ESCs
Extracellular Matrix	ECM
Fetal Bovine Serum	FBS
Hour	h
Hexamethyldisilazane	
Hydroxyapatite	HA
Inductively coupled plasma-mass spectroscopy	ICP-MS
Kilodalton	kDa
Liquid Chromatography – Tandem Mass Spectrometry	LC-MS/MS
Matrix-Assisted Laser Desorption/Ionization	MALDI
Phosphate Buffered Saline	PBS
Polyvinylidene Fluoride	PVDF
Rat Adipose Derived Stem Cells	rADSCs
Room Temperature	RT
Scanning Electron Microscopy	SEM

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis	SDS-PAGE
Stromal Vascular Fraction	SVF
Time of Flight	TOF

Abstract

Skeletal injuries affect millions of people worldwide, making it one of the most common causes of severe chronic pain and physical disability while also being a heavy burden on Australian healthcare, costing approximately $700 million a year. Over the past decades, biodegradable coralline biomaterials have been considered as an alternative implant material for bone regenerative therapy. This is because coralline materials have been found as being clinically advantageous due to their biocompatibility, osteoconductivity and scaffold resorbability. Additionally, coating coralline material with autologous stem cells is desirable for tissue ingrowth to occur rapidly as possible to provide the implant with structural integrity and eventual complete scaffold resorption. Adipose Derived stem cells (ADSCs) are considered promising biological tools for regenerative medicine as they are an accessible and abundant source of stem cells that have shown to be able to differentiate into bone tissue.

Recent *in vivo* and *in vitro* studies of coralline materials seeded with mesenchymal stem cells have produced conflicting results that range from demonstrating complete fracture repair to ineffective tissue regeneration [1-3]. This is because the underlying biological mechanism behind the clinically advantageous properties of coralline material is not well understood.

This PhD project has therefore been developed in order to address the problems outlined above. This work has investigated the effect of seeding rat adipose derived stem cells (rADSCs) and human adipose derived stem cells (hADSCs) onto biomimetic coralline scaffolds. The data presented here demonstrates that ADSCs can be successfully cultured onto coralline scaffolds, which provide a suitable microenvironment for ADSCs to proliferate. Additionally, the research I have undertaken shows that ADSCs seeded on coralline scaffolds undergo a proteomic change that resembles osteogenic cells, without the addition of any external osteoinductive factors.

This project also investigated the effects of different coralline scaffolds such as coralline carbonate, converted coralline hydroxyapatite (cHA), nanoporous cHA, macroporous cHA and high-density cHA on hADSCs where I showed that seeded cHA induced a stronger osteogenic response than seeded coralline calcium carbonate. Furthermore, I identified a unique immunomodulatory response from each seeded coralline scaffold that suggested a microenvironment rich in pro-inflammatory and pro- angiogenic factors which is a physiological feature commonly noted during *in vivo* fracture repair.

Overall this PhD project has contributed significantly to a wealth of biological knowledge about the effects of coralline scaffolds on ADSCs. Future work can utilise what is described here to either fabricate a coralline implant to harness the biological responses we have recorded or apply the data towards a safe and effective animal model for future therapeutic applications.

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1. Introduction

Bone is a highly dynamic, complex and vascularised tissue, which is able to heal and remodel throughout life. At present, clinicians face an enormous challenge in addressing the repair of bone defects due to chronic disease or trauma. According to the current statistics, 2.2 million bone graft procedures are carried out worldwide per year with a steady annual growth of 13% in the number of operative procedures for repairing bone defects in orthopaedics.

Additionally, with an increase in an ageing population in both developed and developing countries, there is a massive global demand for bone graft materials that are capable of rapid bone fusion and recovery times. However, due to the well-known disadvantages associated with autografts (availability of tissue, pain, and morbidity at the donor site and the need to have two operative procedures) and allografts (rejection issues and concerns with disease transmission), synthetic bone grafts, and autologous source of progenitor bone cells are of paramount interest for bone tissue engineering. A fundamental understanding of the physical, chemical, biological, and mechanical processes surrounding the use of biocompatible materials is required to develop bone graft substitute materials.

1.1 Bone diseases epidemiology

Musculoskeletal conditions such as osteoporosis, osteoarthritis, rheumatoid arthritis and skeletal injuries affects millions of people around the world, making it one of the most common causes of severe chronic pain and physical disability [4]. An increase and re-emerging prevalence of injury, the economic and psychological impact on patients remains a persistent problem [5] Musculoskeletal conditions are also a major burden on health systems and social care systems with the number of incidences increasing with an ageing population and higher life expectancy [6]. Furthermore, the United Nations (UN) and World Health Organisation (WHO) recognised that musculoskeletal conditions will be the fourth leading cause of disability by the year 2020 [7]. This led to

a declaration that the period of 2010 – 2020 would be endorsed as the Bone and Joint Decade by raising awareness of an increasing need to supplement the direct and indirect costs of musculoskeletal conditions [6].

1.2 Current treatments

1.2.1 Autografts

Clinically, the current gold standard in cases of significant bone loss such as fracture injury, disease and tumour removal is orthopaedic reconstructive surgery. In these orthopaedic reconstructive procedures, bone grafts are used to promote and support bone tissue regeneration while also providing mechanical support for cellular expansion.

The most common type of bone grafting procedure is an autograft which involves the harvesting of autologous bone tissue from the patient. Autografts have a very high success rate due to the elimination of the risk of tissue rejection therefore cementing the procedure as the gold standard in modern orthopaedic surgery. However, autografts do have their drawbacks, namely donor site morbidity and limited supply [8]. Donor site morbidity is the inability of bone tissue regeneration to occur in the location where the autologous bone was harvested. Such incidences are so common that it has been reported to occur in 44% of cases involving autografting of bone [9]. Donor limitation is a factor frequently encountered in cases of bone disease where the amount of bone that can be harvested is either limited due to impaired healing or even non-existent. Thus, alternative therapies are required and current alternatives to autografts are allografts and tissue orientated scaffolds.

1.2.2 Allografts

Allografts are sourced from serotype matched donor patients and transplanted to the site of injury. Since the tissues are not harvested from the patient, donor site morbidity and limitation do not play a role as drawbacks [10]. Although the use of allografts eliminates the potential disadvantages of autografts, they do have limitations of their

own. Whenever donor tissue is transplanted, there is a chance of disease transmission and chance of rejection [10]. Harsh sterilisation techniques are utilised to limit disease transmission however the procedure can denature the bone matrix and deteriorate its mechanical properties. Furthermore, allografts are susceptible to the hosts' immune system resulting in tissue rejection and ultimately graft failure.

Currently, restorative therapies of bone and joint conditions are commonly performed with autologous and allogeneic transplantations by using autografts and allografts, respectively. However the harvesting and implanting of bone grafts is an expensive procedure which is also at risk of infections at the harvest site and prolonged pain [11]. To limit the use of donor acquired grafts, an alternative source of bone implants has been developed through the process of bone tissue engineering.

1.2.3 Bone tissue engineering

The field of bone tissue engineering has emerged with a goal to bridge this gap between the need for and lack of ideal bone grafts. The implementation of three dimensional scaffolds seeded with stem cells is one such solution. The underlying concept of three dimensional scaffolds is to provide a supportive matrix for cellular growth, differentiation and proliferation in an attempt to enhance tissue repair and regeneration [12], and to achieve these goals scaffold design must consider porosity, degradation rate and mechanical properties [12]. Porosity and uniform pore interconnectivity of the scaffolds are essential to allow infiltration of the cells which provide interconnections between cells for new tissue growth. It has been shown that uniform pore size is crucial *in vivo* as it allows the optimum vascularisation of blood vessels throughout the material [13].

Scaffold designs that have irregular pore dispersion have been shown to cause inflammation of local tissue and gradual tissue damage [14]. The degradation rate of the scaffolds should be comparable to the tissue growth rate, as the implant needs to maintain mechanical properties initially to bear loads, and over time the load bearing

should transfer onto the newly forming bone tissue [15]. The mechanical properties of the scaffold should match the properties of the surrounding tissue so that the role of the bone graft can meet the physical and biochemical function of the tissue it is replacing and/or repairing. Additional attributes that should be considered when discussing bone grafts are biocompatibility, osteoconductivity, osteogenicity and osteoinductivity [16].

Biocompatibility is another essential property required for scaffold bone grafts as it ensures an appropriate host response to the newly grafted tissue construct [17]. Various studies have investigated the biocompatibility of a number of scaffolds *in vitro* and *in vivo,* including hydroxyapatite, bioglass, poly-lactic acid and beta tricalcium phosphate (β-TCP). Plattelli *et al* concluded that the surface chemistry of calcium phosphate materials contributed to their biocompatibility, as the calcium ions leeched from the material was not detrimental to the survival of the host cells *in vitro* and *in vivo*[18]. Host tissue rejection is one of the most common forms of surgery implications in orthopaedics which is why biocompatibility is always tested during scaffold production and implementation [19].

Osteoconductivity is a property which allows native osteoblast attachment while promoting cellular proliferation and migration throughout the scaffold [20]. The attachment can be exacerbated by a nano-porous surface of a scaffold while macro-porosity has been shown to play an important role in the cellular integration as it allows vascularisation of the three dimensional material [21]. These vital components are required for a scaffold to be osteoconductive, and without it, tissue repair cannot efficiently occur.

Osteoinductivity allows a scaffold to possess the necessary bioactive molecules, such as bone morphogenetic protein (BMP), which accelerates osteoinduction in various scaffold materials due to the growth factor being able to induce bone and cartilage differentiation in stem cells [22]. While, osteogenicity allows the established

osteoblasts to produce minerals and calcify the surrounding matrix, which eventually strengthen into bone [23]. These two work in tandem to ensure optimum osteo-differentiation and proper tissue regeneration.

Within this context, bone tissue engineering is now a heavily invested field of research for regenerative medicine. It is an area which incorporates the methodology of tissue formation and attempts to simulate these biological processes outside the body [24]. This involves elucidation of the complex events involved in healing and/or the repair processes involving stem cells in conjunction with the extra cellular matrix to assemble functional units of tissues and organs, an area which has a large commercial and academic interest. The underlying concept of tissue engineering is to incorporate biologically relevant molecules, target cells and a structural scaffold to form a tissue engineering product, which promotes regeneration and repair of tissues. Therefore, bone tissue engineering is a type of tissue engineering which aims to produce composite materials that are either synthetic or naturally derived and have one crucial property, osteoconductivity [24].

1.3 Physiological Process of Bone healing

The process of bone fracture healing restores mechanical function while maintaining the biological mechanisms of the newly formed bone (Table 1.1) (Figure 1.1) [25]. If the process of bone fracture healing occurs without a callus precursor (a fibrous callus containing progenitor cells and fibroblasts to initiate bone healing) then the process is classified as direct or primary bone healing [26]. Direct or primary bone healing allows the cortex of a fractured bone to join together through internal fixation [27]. The osteoclasts and osteoblasts between the fixed fractures bridge the gap with newly formed bone over the remodelling phase [28]. Indirect or secondary bone healing, however, occurs when there is a callus precursor. A callus precursor is formed over four major phases of recovery which involve sequential phases of inflammation, proliferation, migration of osteogenic cells and remodelling of callus to trabecular bones [28].

The inflammation phase is the first phase of secondary bone healing. Blood vessels at the site of fracture constrict to prevent bleeding and within hours the extra-vascular blood cells clot to form a hematoma [29]. The blood cells within the hematoma degenerate and die which release cytokines for fibroblast proliferation. The fibroblasts replicate to form granulation tissues which are aggregated cells with dispersed vascular supply [26]. The vascular vessels allow the next stages of repair to occur.

Proliferation phase involves the migration of periosteal cells to the fracture gap which eventually expand into chondroblasts to form hyaline cartilage [30]. Additional periosteal cells migrate around the granulation site to develop woven bone [31]. The components of woven bone and hyaline cartilage at the site of injury are known as the soft callus [32]. Eventually, the fracture gap is bridged by the hyaline cartilage, connective tissue and woven bone, restoring some of the bones' original strength [26].

Transformation of the soft callus to a hard callus signifies the beginning of the next phase which involves the replacement of hyaline cartilage and woven bone with

lamellar bone [33]. This occurs through the gradual mineralisation of the soft callus with migrating osteoblasts and osteoclasts [26]. Initially, the woven bone is reabsorbed by osteoclasts and substituted with lamellar bone by constructive osteoblasts. Eventually the hyaline cartilage is substituted with lamella bone through the process of endochondral ossification [28]. The lamella bone is dispersed with micro-vessels which support the mineralisation of the callus surface to restore more of the bone's original strength.

Finally, the remodelling process replaces the trabecular bone with compact bone with the aid of osteoclasts which resorb the trabecular bone creating a pit that allows the osteoblast to deposit bone matrix within [26]. Overtime, the remodelling process transforms the hard callus into a fracture callus which is similar to the bone's original shape and strength. The process of remodelling is affected by many biological factors such as age, genetic preposition and environmental conditions [28].

Majority of clinical fractures heal properly and approximately 5–10% of the fractures show impaired healing [34]. Impairment of fracture healing leads to a delay in union of bone and permanent damage of tissues and bone [35]. The goal of therapy for fracture healing is to provide sufficient fracture stability by enhanced surgical and/or non-surgical means. Unstable fractures can also result in delayed healing [34]. Insight to the molecular and biological mechanisms involved in fracture healing can result in the development of new treatment modalities for therapy thus lowering the risks of impaired fracture healing. [28]. Other musculoskeletal disorders are targeted by therapies aiming to enhance bone tissue regeneration.

Table 1.1: Mechanical properties of healthy adult human bone specified by their respective osseous type, Young's modulus, percentage of porosity, compression, flexion and tension resistance [36].

Type of bone	Compression resistance (MPa)	Flexion Resistance (MPa)	Tension Resistance (MPa)	Youngs Modulus (GPa)	Porosity (%)
Lamellar	142-160	151-223	75-180	18-20	5-13
Cortical	130-180	135-193	50-151	12-18	5-13
Spongy	4-12	-	1-5	0.1-0.5	30-90

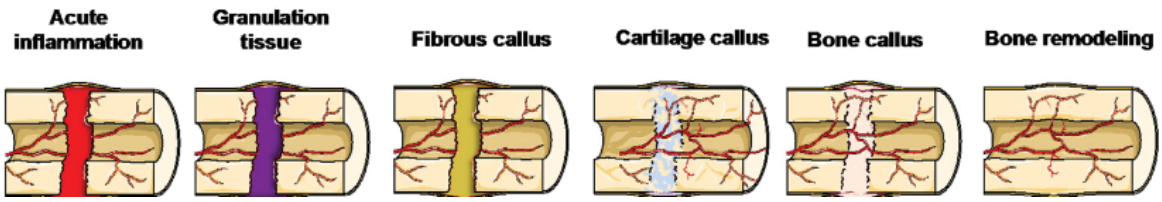


Figure 1.1: The subsequent stages of normal bone healing and repair[37].

The initial phases of acute inflammation and granulation occur during the first couple of weeks after the fracture to form a fibrous callus. Gradually, the fibrous callus breaks down over months to form the later stages of cartilage and bone callus. Finally, bone remodelling occurs over months to strengthen the newly formed bone.

1.4 Biomaterials in Tissue Engineering

Biomaterials are intended to interface with biological systems to evaluate, treat, augment, or replace any tissue, organ, or function of the body [38]. The nature of the interactions between a given biomaterial and its surrounding cells and tissues can be instructive or inductive in stimulating important biological effects. Inductive biomaterials can induce tissue formation with or without the aid of exogenous growth factors implemented within the material [39] while instructive biomaterials stimulate tissue formation through the host's own regenerative mechanisms [40]. Biomaterials differ greatly in comparison to surgical devices as the latter does not intimately associate with local host cells and tissues for improved healing and repair.

Biomaterials gain their advantage where much needed biological actions important in driving tissue regeneration are incorporated within the material as chemical and/or physical cues. These inductive cues have been heavily investigated by many studies in an attempt to dissect the interactions between a material and its surrounding environment [41,42]. This attempt at mimicking biological processes is commonly known as biomimetics and is done so that researchers can modulate the bulk biological properties of biomaterials in other tissue systems to provide external therapeutic scaffolds with dual functions such as drug delivery and three-dimensional matrix cell support. Biomimetic scaffolds aim to enhance cell target functions such as adhesion, proliferation, migration, and tissue differentiation [43].

The architectural properties considered in scaffold designs are porosity, pore size, interconnectivity, and pore-wall microstructures [44]. These architectural considerations are critical for bone healing. Porosity and pore size play critical roles in cell seeding, cell growth and differentiation, and three-dimensional tissue formation [45]. Generally, a pore size between 100 μm and 400 μm is considered to be an optimal dimension to allow interaction with cell and tissue [46]. Interconnected pores allow skeletal and vascular regeneration by acting as channels for cell migration and tissue growth [47]. They facilitate uniform cell seeding, distribution, diffusion of

nutrients to, and metabolites out, from the cell/scaffold constructs. Therefore, high porosity is desirable for bone tissue engineering, however, the mechanical stability of the carrier material must be sufficient to support new bone formation. Since porosity can also affect the polymer degradation rate, to preserve structural integrity and facilitate new bone formation, the scaffold porosity should allow the carrier material biodegradation rate to mimic new bone formation rate. The required architecture of a bone morphogenetic protein (BMP) delivery system for bone tissue engineering is dependent upon on the intended application (e.g., heterotopic ossification, spinal fusion, critical defect healing), therefore, no singular optimal architecture exists. It has been suggested that optimal bone growth requires a pore diameter range of 200–600 μm. In addition, it has been suggested that a porous scaffold supports optimal bone growth by facilitating mass-transport required for cell nutrition and porous channels for cell migration.

This review will cover the two major classes of biomaterials, polymers and ceramics, in terms of their fabrication, industry application and relevance in clinical practice within the context of bone tissue engineering.

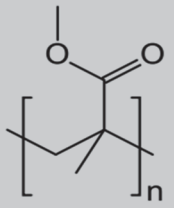
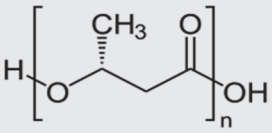
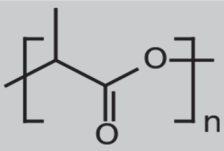
1.4.1 Polymers

Polymers are composed of heterogeneous macromolecules that are typically derived of repeating subunits bonded along the length of a molecule [48]. The type of macromolecule utilised determines the property of the polymer, a number of which have already been manufactured in the field of biomaterials. Poly(methyl methacrylate), also known as PMMA, is a shatter resistant acrylic glass which has various industrial and clinical applications [49]. In its liquid state, PMMA is used as a type of ‘bone cement’ in traumatic skull defects. The accessibility of PMMA components gives it an economic advantage over other polymers and it can be easily molded during fabrication. The covalent cross-linking of monomers characteristic to polymers gives the biomaterial considerable strength while preventing biodegradation. Non-resorbable biomaterials are ideal for permanent and long term surgical implants however it can sometimes discourage the regrowth of host tissue by preventing

natural meshwork to occur over the target site [50]. A biodegradable polymer is desired for stable biological integration as it allows the formation of natural bone to take over the healing site initially assisted by a scaffold construct.

The production of biodegradable polymers is emerging in the field of biomaterials, one of which are Poly(hydroxyalkanoate)s (PHAs) [50]. PHA is a class of biodegradable and biocompatible thermoplastic polymers produced by various microorganisms such as *Pseudomonas*, *Oleovorans* [51], blue-green algae, and some genetically-modified plants [52]. Copolymers of PHA are utilised in various ratios to tailor the physical and chemical characteristics required for their individual purposes which vary in their application. Studies have already demonstrated that some types of PHA are biocompatible with epithelial cells, osteoblasts and bovine chondrocyte cell lines *in vitro* [53] suggesting they could have an application as a tissue scaffold. However, the limitations of PHAs, such as structural fragility, low abundance, expense and difficulty of production, has limited their potential utilisation as a novel biomimetic scaffold.

Table 1.2: Commonly used polymers in the field of bone tissue engineering.

Polymer	IUPAC structure	Example
Poly(methyl methacrylate) (PMMA)		Acrylic glass, Bone cement.
Poly(hydroxyalkanoate)s (PHAs)		Bone plates.
Polylactic acid (PLA)		Drug delivery systems

1.4.2 Ceramics

Ceramics are a type of material which consists of crystalline metallic oxides, carbides, nitrides and borides fused by sintering, a process involving fabrication at high temperatures. The properties of ceramics include low electrical and heat conductivity, brittleness and they are known to elicit very little tissue reactions [54]. Certain ceramic compositions are considered to be bioactive, a property which allows the bone to chemically bond to the material [52]. An example of one such commercialised ceramic composition is bioglass, which is composed of SiO_2, Na_2O, CaO and P_2O_5. The fabrication of bioglass is performed through conventional glass manufacturing protocols and even minor changes in their composition can alter the bioactivity of the material [55]. Furthermore, bioglass has shown to be osteoconductive and osteoinductive[56,57], allowing the biomaterial to be classified as a 'class A' bioactive material [58]. Other similar commercially available bioceramics are listed in table 1.3 and 1.4. Clinical reconstruction of orbital walls and facial bone defects have utilised granular and solid forms of bioactive glasses [59]. Bioactive glasses are osteo-compatible and the infection rate after implant is low.

Another group of ceramics important for bone tissue engineering are comprised of materials deriving from calcium. Such ceramics are composed of calcium sulphate, calcium phosphate and calcium carbonate derivatives and their structural conformity differ in terms of density, porosity and granules [60].

Table 1.3. Ceramics commonly used in biomedical applications catergorised based on their production cost, biocompatibility, biodegradability and functional bone strength**.**

Ceramic	Biocompatible	Structural bone strength	Biodegradable	Production costs
Beta-tricalcium phosphate	Yes	High	Yes	Low
Calcium carbonate	Yes	High	Inconsistent	Low
Bioglass	N/A (inert)	Weak	N/A	Low
Hydroxyapatite	Yes	High	Yes	Low
Pyrolytic carbon	No	High	No	Low
Alumina	Yes	High	No	Low
Zirconia	Yes	High	No	High

Table 1.4 Mechanical properties of bioceramics.

Ceramic	Compression Strength (MPa)	Porosity (%)	Flexion Resistance	Tension Resistance	Youngs Modulus
Tricalcium phosphate	580-910	50	170-228	43-75	80-120
Calcium carbonate	745-923	80	112-135	Variable	50-73
Bioglass	500	5	N/A	56-83	22
Hydroxyapatite	510-896	90	160-189	69-193	40-117

1.4.3.1 Calcium ceramics

1.4.3.1.1 Calcium carbonate (CC)

Calcium carbonate ($CaCO_3$) is a biocompatible and osteoconductive material but it does not have any osteoinductive properties. The resorption rate for CCs is high however animal models have shown that the resorption rates can vary from days to only a few weeks [61,62]. Initial studies on CC began in the early 1970s and involved animal models of cancellous defects in dogs and properties such as biocompatibility was found in CC ceramics which allowed infiltration of bone cells within pores [63]. As research progressed, bioactivity was also observed as the CC were resorbed and replaced with new bone after one year. Bioactivity continued to be observed in different animal models such as sheep and pigs weeks after implantation [64]. After successful animal models, clinical trials were attempted and published in France by the Intitut de Rechrches Orthopediques during 1980 [65]. Clinical applications of CC included maxillofacial surgery to correct periodontal defects [66] and to fill and reconstruct bony defects requiring cranial surgery [67]. Further clinical applications include filling of fractures in lower limb metaphysical injuries and tibial osteotomies [68]. Currently, CC can be used to lessen the number of bone grafts and autogenous bone required for spinal fusion therapy [69].

Few studies have investigated the molecular mechanisms behind bone growth in CC implants. The reason for such a gap in the literature is due to the lack of experimental protocols being able to measure the partially resorbed implant of CCs. Even though fibrous tissue and bone do grow inside CC pores, the experimental evidence to show this has been scarce. Furthermore, varying indications of resorption of CC implants between different animal models indicates a lack of understanding of the effect of biomaterials on its target tissue site [70]. For example, Guillemin *et al* implanted CCs in the femur cortex of sheep and found that after 1 month, 93% of the CC blocks were resorbed while in pigs only 64% were found to be completely resorbed [64]. Another variation of CC resorption was shown in humans where granular CC implants were not completely resorbed after several years while it only took 24 weeks to resorb in pig connective tissue [71]. One reason for this variation is differences in the expression

level of the enzyme carbonic anhydrase which plays a key role in the resorption process and is abundant in osteoclasts. Within the site of CC application, the enzyme is able to lower the pH which begins to dissolve the CC implant [64]. The expression of carbonic anhydrase differs between species, thus different resorption times were observed. It should be noted that for efficient resorption of the CC scaffold, direct contact between the cells and the ceramic matrix is required [64].

1.4.3.2 Calcium sulphate

In 1892, calcium sulphate was one of the first ceramics implemented as a bone substitute. The material was used to fill defects in the bone of patients which was allowed to heal over time [72]. As research progressed, the properties of calcium sulphate were shown to include a high degree of biocompatibility and bioactivity however the material is not osteo-inductive [73]. Calcium sulphate has a fast resorption time which can act as a limitation by not allowing bone formation to occur [74]. In conjunction with other limitations, such as poor mechanical properties, has reduced the application of calcium sulphate as a bone substitute. The fast resorption can allow calcium sulphate to become an efficient drug delivery system where a faster degradation time is favourable. These drug delivery applications of calcium sulphate have already been implemented in some studies [74].

1.4.3.3 Coralline based ceramics.

Not all ceramics are synthetically produced as natural sourced ceramics such as coralline based ceramics have been extensively utilised in the field of bone tissue engineering. [75,76]. Coralline ceramics are commonly used as a bone substitute because they have a porous exoskeleton structure similar to mammalian bone [64]. The exoskeleton of corals arises from the formation of coral reefs colonies. The sizes of these colonies vary between species and range from one millimetre to several centimetres [77]. The coral polyp colonies share a symbiotic relationship with unicellular algae that photosynthesise essential compounds for the polyps [78]. The matrix of the coral skeleton is made through the calcification of a substance, secreted by the outer layer of a polyp in seawater. This build-up of calcification material allows

the polyp to live in the upper part of the coral skeleton which ultimately leaves an empty skeleton behind at the bottom.

The application of coralline based ceramics have been implemented as bone grafts on a number of genera which include Acropora, Montipora, Turbinaria and Porites [76] [36]. The genera Porites have the most potential as a bone substitute as it forms large colonies which are common throughout the Atlantic sea. The corals from this genus contain pore sizes ranging between 100µm - 150µm and the pores interconnect with each other. Due to its pore size, most corals from the genera Porites have been commonly utilised as a bioceramic due to its calcium and phosphate composition as it meets the structural requirements of bone growth [79].

1.4.3.4 Calcium phosphate

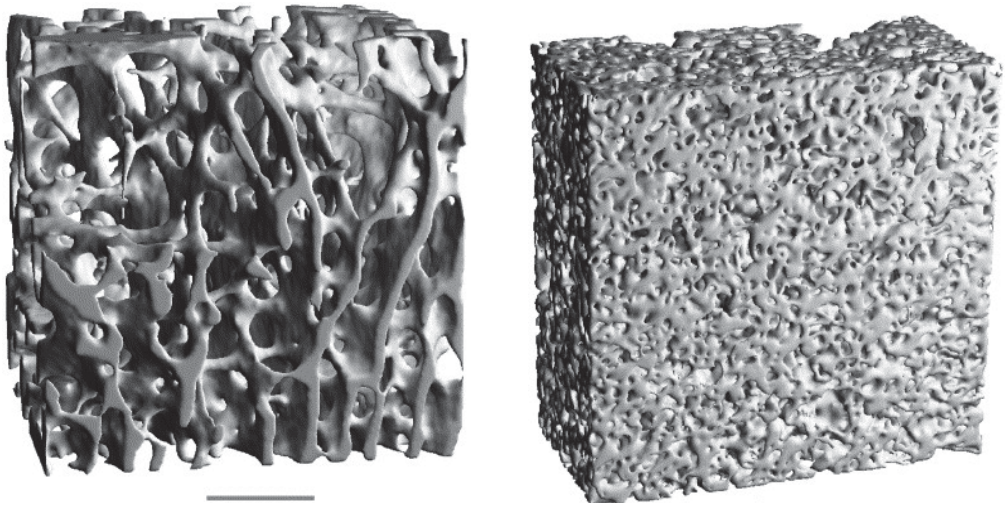
There are two main types of calcium phosphate materials currently used in tissue engineering, tri-calcium phosphate (TCP) $Ca_3(PO_4)_2$ and hydroxyapatite (HA) $Ca_{10}(PO_4)(OH)_2$. Hydroxyapatite is known to be bioactive, biocompatible and osteoconductive. Tri-calcium phosphate also has the same properties however it has a faster *in vivo* degradation rate than HA [80,81]. Calcium ceramics share a similar physical and chemical structure to bone [68]. This is partly because the synthetic form of HA is shown to be chemically similar to the natural main inorganic salt of bone also known as hydroxyapatite [82].

Between the different types of calcium phosphate, HA has been heavily investigated with clinical model studies dating back to the 1970's [83]. Porous HA is favoured over dense HA as porous HA allows better integration of the bone. When HA has been placed into bony defects, bone growth into pores has been observed through changes in mechanical measurements, a correlation well established in the field of bone engineering [84].

There are several methodologies that can currently manufacture porous HA, the homogenisation of calcium phosphate powder with appropriately sized naphthalene particles being one of them. This results in a macroporous material after the

naphthalene has been removed. The macroporous material is sintered at high temperatures between 1000ºC - 1300ºC to achieve a brittle final product [85]. Alternatives to this methodology do exist and they rely on the decomposition of hydrogen peroxide instead of naphthalene to generate a pore-filled structure [86]. However, the final macroporous products achieved from both methodologies were not consistent in their dispersion of pores [86]. To avoid this problem, a completely different approach was developed in the early 1970s which was based on harvesting the structure of certain reef-building coral species which naturally resembled evacuated osteon bone [87]. The coral pore size is consistent and has shown to be uniform just like other types of porous HA as shown in Figure 1.2 which compares the uniformity of pores between HA and trabecular bone through Microtomography CT scan (MicroCT). Uniform pore sizes have been previously found to allow regrown cortical bone to proliferate on the outer part of the structure and a trabecular like bone marrow to be formed on the inside [88].

To achieve uniform and controlled porosity between each manufactured specimen, the 'replamineform process' was developed in 1972 by White et al [63]. The 'replamineform process' is the favoured methodology for the fabrication of coralline porous materials as it allows uniformity of pore sizes between each specimen which can differ depending upon particular coral species precursor. The process allows complete uniformity of interconnecting pores throughout the material and produces an accurate translation of bone mechanical properties due to the materials microstructure ratios [79]. Additionally. the replamineform process allows coral precursors to have its physical configuration preserved while tolerating its chemical composition to be changed [79]. This was further refined to produce a HA implant with controlled porosity but with a chemical composition differing from that of the original coral material through the hydrothermal exchange reaction where the calcium carbonate ($CaCO_3$) skeleton of coral is turned into hydroxyapatite.



(A) **(B)**

Figure 1.2: A comparison of pore morphology between (A) coral sourced hydroxyapatite and (B) trabecular bone using MicroCT, illustrating the similar architectural resemblance between the two structures**.** [89]**.**

Several studies from 30 years ago have shown that porous hydroxyapatite is osteoconductive, biocompatible and very inert [88,90] as it has limited osteoinductive properties [91,92] with an *in vivo* study concluding that the HA inhibits osteoinduction of the bone matrix [93]. Osteoinductive properties can be achieved for hydroxyapatite through chemical induction via slow chemical delivery of bone morphogenic protein -2 (BMP-2) as shown by Xiong and colleagues [94]. BMP2 is a potent biochemical stimulus for *in vivo* bone regeneration as it induces a signal for the Wnt/β-catenin signalling pathway[95]. However, neither of the aforementioned studies further pursued a proteomic nor genomic analysis of the HA implanted tissues.

The restorability degradation rate of HA is slow [96] and HA has been clinically applied in dental, craniofacial and orthopaedic surgery, mainly in block form [97]. A pliable paste forms of HA, known as hydroxyapatite cement was developed for cranial defects. The HA cement could be moulded into any desired shape and the material integrates with the host bone and is claimed to be replaced by bone over time [98,99].

Due to the brittleness of porous HA, its application as a surgical implant has been limited to non-loading sites [73]. However, if seeded with bone forming cells, its compressive strength was enhanced by ingrowth of bone which was comparable to that of cancellous bone [100]. Porous HA has a slow resorption however this delayed resorption is considered advantageous as a long term drug delivery system. HA is considered an ideal biomaterial as it is osteoconductive, biocompatible, easy to shape; readily biodegradable and resorbable after calcification of new bone, and can be economically manufactured [86]. Its mechanical properties can be comparable to those of cortical bone [86]. When used *in vivo*, calcium phosphate particles both embedded on the surface and inside of the bioceramic allowed for improved tissue integration through pH buffer exchange and providing a suitable microenvironment that mimics the host tissue's inorganic phase [24,101,102], which is why ceramics are favoured by bone engineers.

However, the main drawback of polymer and ceramic based composites is difficulties in forming uniform pore sizes through the scaffold matrix. It has been shown that uniform pore sizes are crucial *in vivo* as it allows the optimum vascularisation of blood vessels throughout the material which eventually promotes osteo-conductivity [24,103]. The composites of HA contain these uniform pore sizes within their structure and with the above mentioned biological and mechanical properties of ceramics, HA is seen as an ideal scaffold in the field of bone tissue engineering [19,104]. Furthermore, these scaffolds can be further processed into any shape while also allowing incorporation of other bioactive substances such as strontium [105], transforming growth factor beta-1 (TGF-β1) [106], bone morphogenetic proteins (BMPs) [107], and statins [108].

1.5 Stem cells and tissue regeneration

There are two components essential in a bone tissue engineering construct, a scaffold, as previously discussed and regenerative cells utilised to enhance the regrowth of tissue morbidity. The types of cells commonly applied in the field of bone tissue engineering will be discussed in this section.

1.5.1 Embryonic Stem Cells

Embryonic stem cells (ESC) were first isolated as a cell line in 1998 from human blastocysts [109], a fertilised human embryo that contains an inner cell mass consisting of 50-150 embryoblasts. The isolation procedure of ESCs involves the loss of the human embryo and has resulted in controversy as some believe that human life begins at fertilisation and its extraction is considered as a loss of life. These ethical debates have hindered ESCs implementation as a research and/or therapeutic tool.

The reason that ESCs are pursued by researchers is because of their ability to differentiate into any of the three germal layers, a characteristic known as pluripotency which has great potential as a tissue engineering device. However, due to the previously mentioned ethical issues, different countries and states differ in the laws and regulation of embryonic stem cell research. Currently in Australia, research on human embryos is regulated by the 'Human Embryos Act 2002' and 'Human Embryo Research Amendment Act 2006'.

Under both of these acts, a researcher is allowed to extract ESCs from excess human '*In Vitro* Fertilised (IVF)' embryos with legal consent from the donor without any financial reimbursement involved. The law requires that any research involving ESCs must require a license from the National Health and Medical Research Council (NHMRC) and any cell line obtained from IVF are still subjected to any relevant guidelines governed by the NHMRC. The legislation has not surprisingly created limited sources of ESCs. Alternate sources of stem cell lines have been discovered, including adult stem cells, which don't have the ethical limitations of ESCs as it does not require

death of the donor cell. Adult stem cells have created an alternative source of stem cells which are more abundant, ethically sound and easier to obtain than ESCs [110].

1.5.2 Adult Stem cells

Adult stem cells are dispersed throughout the tissues of animals and are defined by two essential properties (i) self-renewal, which allow the cells to go through continuous multiple cycles of cell division while retaining their ability to remain in a basal undifferentiated state and (ii) multi-lineage potency which is the ability to differentiate into specialised cell types [111]. There are various types of differentiation potency for stem cells and they include: (i) totipotency which is the capacity to differentiate into embryonic and extra-embryonic cell lineages and have the potential to differentiate into a complete and viable multicellular organism [112]; (ii) pluripotency which is the ability to differentiate into nearly all cells derived from any of the three germ layers [43]; and (iii) multipotency which refers to a restricted closely related family of cells that can differentiate into cells of related respective lineage [43]. Adult stem cells are multipotent stem cells which play an important role in the body by maintaining tissue repair, replenishing specialised cells and maintaining normal turnover of cells in regenerative organs [113].

1.5.3 Mesenchymal stem cells

Mesenchymal stem cells (MSC) are a type of multipotent somatic stem cells derived from the mesodermal layer of tissues which, under the appropriate signalling molecules and microenvironment, can differentiate into myocytes, osteocytes or even cells of non-mesodermal derivation such as neuronal cells and hepatocytes (Figure 1.3) [114]. The differentiation of MSCs can be induced *in vitro* by a number of already published protocols (Table 1.6)[115]. MSCs were first isolated from the bone marrow and were hence termed ‘marrow stromal stem cells’. However similar populations of multipotent stem cells have been discovered in other tissues which meant that the nomenclature of these cells could not be based on their site of isolation [116]. MSCs have been isolated from other stromal compartments of adipose tissue, umbilical cord blood, peripheral blood, connective tissues of the dermis and skeletal muscle [117].

MSCs have been defined by their plastic adherent growth *in vitro* and subsequent expansion under specific culture conditions, by a panel of surface antigens and by their *in vitro* and *in vivo* differentiation potential. Induction of differentiation into osteoblasts, adipocytes, and chondrocytes under appropriate culture conditions has been demonstrated extensively [118-120]. Phenotypically MSCs have been defined as being positive for the surface markers CD29D, CD44D, CD90D, and CD105D [121,122].

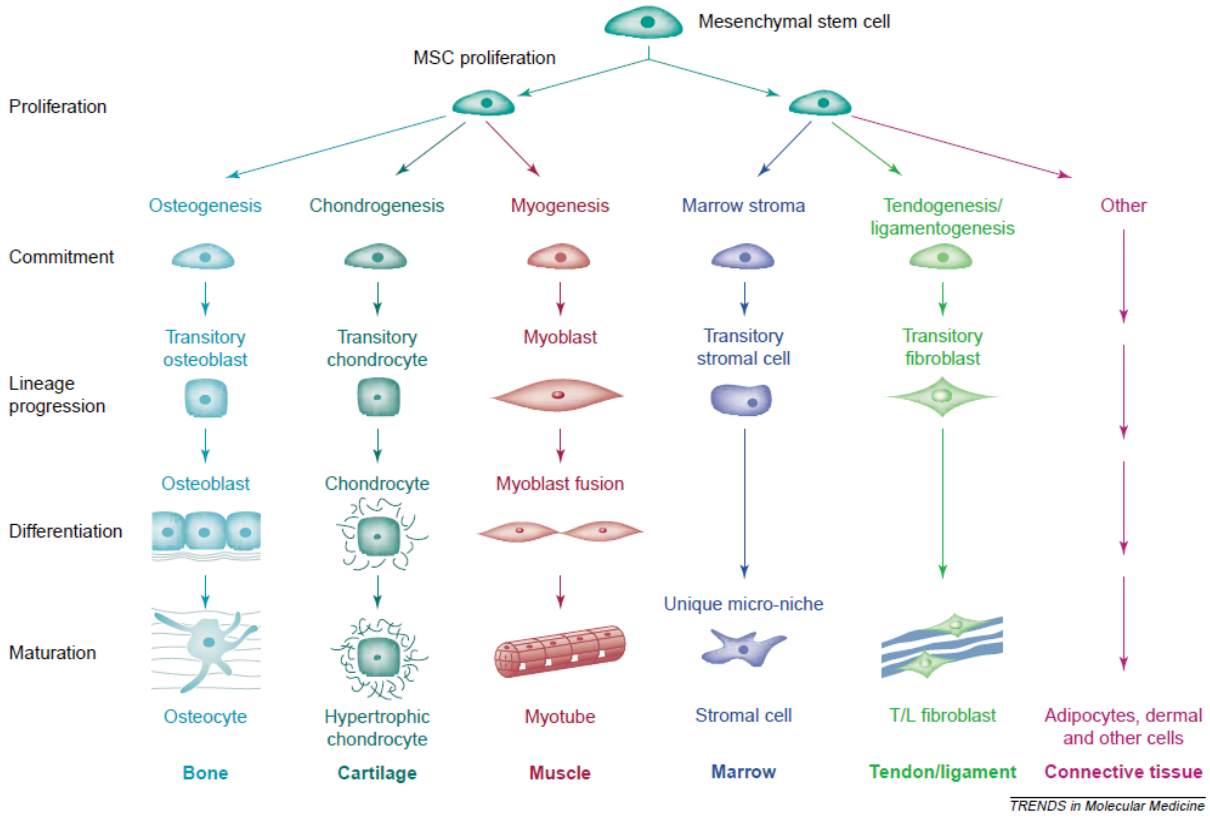


Figure 1.3: A schematic diagram showing levels of cellular transitions between each differentiation state for mesenchymal stem cells.

The diagram represents the main cell types involved during trans-differentiation and other cells involved in between each lineage step may not be present in the diagram. Image is sourced from Caplan and Bruder, 2001 [123].

MSCs are considered good candidates for cellular therapy in an allogeneic setting as it has been observed that MSCS do not express co-stimulatory and MHC class II molecules required for immune detection and thus can escape the hosts' immune response [124]. MSCs have also been considered as a re-infusion therapeutic for chemo-therapy patients. The potential therapeutic application of MSCs as an re-infusion target was observed in a study where the total body irradiation of mouse models induced an increase in MSCs colonisation levels in the various organs and tissues [125].

Such a finding indicates that MSCs are capable of 'homing' into target sites to initiate repair and healing processes. However, in the literature, there is a lack of reports of effective and reliable methods to deliver MSCs to target organs and tissues to initiate site regeneration and repair [126]. A biomimetic scaffold seeded with MSCs can readily apply the regenerative cell to target sites, a possible solution to the issue raised by Bianco *et al*., [126] and subsequent characterisation of its implementation is one of the aims of this research project. Due to their accessibility, expandability, and multi potentiality, MSCs hold promise for clinical applications [121]. A precise characterisation of MSCs intended for human use thus represents an essential requirement for future development and for exploitation of stem cell research in clinical applications.

1.5.4 Bone Marrow Derived Stem Cells.

Bone marrow derived stem cells (BMSCs) are a type of MSC found on the stromal layer of the bone marrow [127]. Previously, BMSCs were known by two interchangeable terms, Mesenchymal Stem Cells and Stromal Stem Cells (SSC) [128]. Most of the earlier MSCs nomenclature were based on initial reports of its site of occurrence (e.g the bone marrow) and the lack of discovery of other types of MSCs at the time. This meant that neither of the earlier terms was sufficient enough to describe BMSCs since MSC implied lineage to the mesoderm which is not true for BMSCs as they cannot differentiate into hematopoietic cells [50], a property restricted to hematopoietic stem cells [129].

Furthermore, the reports of other types of MSCs being found in the stromal compartments of other tissues including adipose and umbilical cord meant that the term SSC could no longer be used as a term to describe BMSCs [130]. Being a type of MSCs, BMSCs are multipotent stem cells that are known to differentiate into osteocytes, chondrocytes, myocytes, stromal cells, fibroblast and adipocytes [131]. The clinical eligibility of BMSCs in comparison to Adipose derived stem cells (ADSCs) will be expanded further in a later section of this review.

1.5.5 Adipose Derived Stem Cells

ADSCs are mesenchymal stem cells that are sourced from the adipose tissue and possess a multi-lineage potential similar to mesenchymal stem cells [132]. ADSCs are capable of multiple mesodermal lineage differentiation such as bone, cartilage, fat, and muscle while also retaining the capability to differentiate into non-mesodermal lineage cells such as hepatocytes [116,133,134]. In addition, ADSCs can also be induced to differentiate into neurogenic cells, [135], indicating an ectodermal differentiation potential. This mesodermal and ectodermal capacity means that adipose tissue represents a source of adult stem cells that can be utilised in the field of regenerative and replacement medicine. ADSCs are phenotypically similar to MSCs as they express CD29, CD44, CD71, CD90, CD105/SH2, and SH3 [136,137]. ADSCs can be somewhat distinguished from MSCs by examining the expression of CD106 which is only positive in MSCs [138]. The absence of CD106 on ADSCs is consistent with the characterisation of these cells as a non-hematopoietic tissue.

Adipose-derived stem cells can be sourced from human fat, usually by liposuction [139]. Human adipose-derived stem cells have also been shown to differentiate into bone, cartilage, fat, and muscle, which make them a possible source for future applications in regenerative medicine [9]. Furthermore, there are no immunological barriers for these cell types as they are isolated from the recipient and are considered as an autograft [116]. This reduces the risk of tissue rejection and infection while, with the ease of harvesting ADSCs, site morbidity and low cell counts are also limited. Such

ideal characteristics are why ADSCs are attracting commercial and academic interest in the field of tissue engineering.

1.5.6 BMSCs and ADSCs as a source of mesenchymal stem cell for tissue engineering

BMSC and ADSC both exhibit a fibroblast like cell morphology analogous to MSC under light microscopy which is characterised by a small cell body with a few cell processes that are long and thin [140]. They are phenotypically similar by being positive for the known MSC markers CD73, CD44, CD105 and CD90 and negative for the haematopoietic marker CD45 [138]. They differ in their expression of CD106 which is a positive surface antigen only for BMSC and negative for ADSC while ADSC is positive for CD34 and negative for CD106 [140]. A study comparing the expressed proteome of ADSC and BMSC using 2D electrophoresis, detected that 18% of the fractionated protein spots were found to be differentially expressed between ADSC and MSC [141]. The proteins in the protein spots, however, were not identified. The different proteomic signatures could have occurred due to the different protein extraction protocols utilised for both cell sources which is known to play a role in inducing a different proteomic expression [141]. The identification of these proteins could prove to be useful in understanding how differing extraction protocols can affect the proteome of a MSC.

Differentiation pathways common to ADSC and BMSC have been previously identified through a transcriptomic study which found that ADSCs preferentially differentiate into adipose cells while BMSC preferentially differentiate into bone and cartilage cells [142]. Other studies have also demonstrated that the expression of the transcripts for osteogenic proteins osterix and osteocalcin were significantly higher in undifferentiated BMSCs compared to undifferentiated ADSCs [143]. Therefore it is well known that both BMSCs and ADSCs can undergo the same lineage differentiation potency however they do so through different molecular mechanisms. Furthermore, BMSCs are more prone to osteogenic differentiation than those ADSCs [144]. Such a finding indicates that BMSCs to be a much more preferential mesenchymal stem cell for bone tissue engineering compared to ADSCs, however ADSCs are more abundant

than BMSCs as it is one of the richest sources of MSCs [145]. The stem cell incidence in bone marrow is estimated to be about 1 per 10^5 cells [146] while 2–6 per 10^8 ADSCS can be obtained from only 300ml of adipose tissue [147]. The acquisition of adipose tissue is much less expensive and less invasive than bone marrow and the yield of stromal cells as previously mentioned is much greater in adipose tissue. Thus, in the perspective of cell therapy, adipose tissue represents an abundant, practical, and appealing source of donor tissue for autologous cell replacement.

1.6 Proteomics

There is a rapidly growing need to understand how mesenchymal stem cells are affected by bioactive materials from a systems biology perspective which involves the incorporation of genomic and proteomic analysis as a whole interactional network [148,149]. This differs from a reductionist perspective which often too simplistically characterises molecular mechanisms. There is a greater need to go beyond the restrictive assessments of previously characterised key proteins using flow cytometry (e.g cytoskeleton or phenotypical marker proteins) and considering the whole cell response in order to discover which cellular pathways are important in processes such as material related bone differentiation [150]. Current investigations have started taking a transcriptomic approach using RNA microarrays [122]. However, genomic data considers the cell's response before essential biological steps, such as protein translation and post-translational modifications, which can affect the predicted gene regulatory outcome [151]. Thus, a proteomic approach is complementary.

Proteomics is the study of the complete set of proteins expressed in a given cell or tissue under a defined set of environmental conditions [152]. It is defined as "the use of quantitative protein-level measurements of gene expression to characterize biological processes and decipher the mechanisms of gene expression control" [153] and attempts to characterise proteins in terms of their location, function, structure interactions, subcellular distributions and activity [154] to understand phenotype and cellular behaviour [154]. Correct bioinformatic prediction of Open Reading Frames (ORFs) is still a problem however one arm of proteomics known as proteogenomics aims to improve gene annotations by using proteomic information to determine a protein's cleavage sites and post transitional modifications.

This has led to a spark in proteomic research during the post-genomic era as the genomes of many model organisms have been extensively sequenced [155]. However, this does not imply that genomic information can be disregarded, instead mass spectrometry (MS) data of peptide fragmentation is searched using amino acid

sequences thus MS based proteomics relies on genomics. It should be understood that the old dogma of 'one gene equals one protein' is no longer true due to a number downstream modifications that occur on proteins as previously mentioned. Thus it is essential that proteomics to be used in tandem with genomics and other methodologies to give a thorough understanding of a given cellular system.

1.6.1 Proteomics Methodologies

Currently, proteomic research can be broadly divided into protein-centric and peptide-centric approaches. A protein-centric approach commonly involves fractionation by electrophoresis which separates complex mixtures of intact proteins using molecular mass and charge, preserving as much of the protein cleavage fragments and isoforms as possible. This approach keeps peptides from the same protein in the same sample during MS analysis. Protein fractionation can occur either through 1-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS PAGE) or 2-dimensional polyacrylamide gel electrophoresis (2D PAGE) [156]. Proteins have been separated by electrophoresis in polyacrylamide gels for more than 40 years and is utilised to separate and characterise mixtures of biological samples in terms electrophoric mobility [157].

The advantage of SDS-PAGE is that it allows the solubilisation of hydrophilic and hydrophobic proteins through the surfactant sodium dodecyl sulfate (SDS). SDS is a strong ionic detergent which denatures non-covalent bonds in proteins allowing solubilisation and loss of native conformity while coating the structure with a constant negative charge. This constant negative charge helps in the migration of proteins during electrophoresis. While 1D SDS PAGE is based upon separation of samples on molecular weight, 2D PAGE separates proteins on isoelectric point (pI) and molecular weight. The reproducibility of 2D PAGE, however, is difficult as slight changes in the running conditions or gel can lead to artefactual errors [158]. 2D-PAGE can be analysed by DIGE whereby the proteins are fluorescently labelled with fluorophores of different wavelength and run in the same 2D gel and compared [159] .

This methodology utilises 300 µg of protein to be utilised for 3 replicate gels to able to retain sensitivity [160]. Peptide-centric approaches, where all proteins in a sample are immediately digested to peptides, reduces complexity of samples using chromatography however the peptides can become disconnected from their parent protein making it difficult to determine presence of protein isoforms and cleavage fragments during MS analysis. The advantage of the peptide-centric approach is that the sensitivity and resolution of reversed phase chromatography and mass spectrometry is significantly increased and a greater depth of proteome coverage can be achieved at the expense of individual protein sequence coverage.

All proteomic samples undergo analysis using MS. MS allows the measurement of the mass to charge ratio (m/z) of a given molecule and its fragments. The mass spectrometer has been used in the field of physics since the early 20th century however it wasn't until 1997 when it was first implemented in the field of proteomics [153]. To achieve optimal proteome coverage using MS, two main factors need to be considered; dynamic range and sample complexity [157]. Dynamic range refers to the difference in protein concentration between high and low abundant proteins while sample complexity is the number of different proteins that are present in a sample and their relative individual concentration [161].

In MS, pre-fractionation of protein and peptide samples is essential to optimise dynamic range (*i.e.* enabling low abundant proteins to be detectable at the same level as high abundant proteins) by simplifying the complexity of samples [161]. Fractionation is commonly followed by or used in tandem with enzymatic digestion which cleaves known sites of the protein backbone to yield peptide fragments; therein complementing the goal of reducing sample complexity [162]. The data obtained from MS (*i.e.* the m/z ratio and spectra) is searched through numerous databases using a search engine such as MASCOT which uses an algorithm to allocate the peaks in a mass spectra determined from the fragmentation during MS and matches it to theoretical peptide fragment masses derived from a protein sequence to identify a protein [154]. The experimental design of proteomic studies are dependent upon the type of data

desired, be it qualitative or quantitative; however no single or tandem forms of methodologies will yield a simple solution as proteins are highly complex and diverse samples in comparison to RNA/DNA samples.

A number of technologies have emerged that provide a powerful set of tools to globally study and quantitate the differences in proteome expression under different conditions [156]. These include protein labelling technologies such as isobaric tag for relative and absolute quantitation (iTRAQ) and stable isotope labelling with amino acids in cell culture (SILAC), which can be incorporated with 1D SDS PAGE and liquid chromatography-mass spectrometry (LC/MS) as a quantitative instrument and 2D PAGE which may also incorporate MS for protein identification but uses densitometry and differential in-gel electrophoresis (DIGE) as a quantitation tool [160].

iTRAQ involves the chemical labelling of side chain amines of lysine and at the N-terminus of tryptic peptides of the proteome with tags of varying mass [161]. This allows labelling of all peptides and post MS acquisition quantitation of different treatments of the sample. The samples are then pooled and fractionated by liquid chromatography before analysis by MS. The advantage of iTRAQ is that it reduces analysis time by allowing the parallel proteomic analysis of multiple samples, all of which can be genetically identical cell lines but subjected to different environmental conditions that may change the proteome [161]. However, iTRAQ data can be difficult to interpret due to the very large number of results often achieved and thus important data can be difficult to dissect out [162]. Furthermore, the reagents involved in iTRAQ are costly thus limiting its application at running biological replicates but can be economical as less technical replicates need to be performed, reducing instrument analysis time [162].

SILAC involves the cultivation of cell lines in the presence or absence of stable isotope labelled amino acids. One of the cell culture mediums contains normal amino acids while the other contains amino acids labelled with non-radioactive isotopes typically 13C lysine where all carbon atoms in the amino acid are tagged at 13C [115]. These

amino acids are incorporated into the proteins of the cells during their metabolic growth in culture. These samples are then pooled and fractionated before analysis by tandem mass spectrometry (MS/MS) [115]. SILAC is however, restricted to cells that can be grown in culture and is not useful in systems where harvesting of tissue is required. Those samples are generally analysed by iTRAQ [163].

1.6.2 Current Proteomic Investigations into Mesenchymal Stem cells

Various studies have attempted to uncover the molecular mechanisms underlying the commitment process of mesenchymal stem cells by studying their differentiation into osteoblasts, neuronal cells, chrondrocytes, myocytes and adipocytes [115,163-165]. Within the first era of research on mesenchymal stem cell differentiation, the first goal was to determine if there was a change in the expressed proteome between the undifferentiated and differentiated state of the cells, some of which has been listed in Table 1.5. In the case of osteo-differention, Salasznyk *et al* compared human ADSCs with human osteoblasts using 2D PAGE and differentially displayed proteins were identified using tandem MS. The study concluded that a significant shift in the proteomic profile occurred during MSC differentiation due to 65% of the protein spots being unique in the osteoblast proteomic profile. The proteomic profile also suggested that the ADSCs contained mostly high abundant proteins in comparison to the osteoblast profile which comprised of mainly low abundant proteins. This was further supported by their transcriptomic study [166]. This occurred because the basal cell line expressed a wide array of abundant proteins before signaling for differentiation and once a state of differentiation was achieved, a more specialised repertoire of proteins began to be expressed [122]. However, the study did not attempt to induce differentiation to their human ADSC cell line *in vitro*. Instead, the authors purchased a prepared specialised line of osteoblasts. This could be an issue as batch to batch variation between the same commercialised cell lines of MSCs do exist [167]. Overall, based on these first studies (Table 1.5), it appears that it is very difficult to identify a proteomic signature specific for MSCs [168].

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Table 1.5: Summary of published mesenchymal stem cell proteomic studies based on the year of publication, type of proteomic methodology utilised and results obtained. This is only a collection of studies that have been covered in this review.

Year of publication	Investigation	Methodology	Results
2008**[162]**	Identify changes in the proteome between diseased (osteoporosis) and normal cell state in BMMSCs	2D-DIGE SPOT EXCISION and MALDI/MS/MS	14 metabolic enzyme proteins, 12 motility proteins 3 transporter proteins Found in healthy patients but not in osteoporosis BMSCs.
2008**[169]**	To quantitatively compare ADSC and BMSC proteome *in vitro*	2D SDS PAGE Spot detection and quantification	Despite a general similar proteomic profile between ADSC and BMSC, independent samples of ADSC were more related together rather than to BMSC.
2009 **[170]**	To compare the phenotypic expression of ADSC and BMSC proteome *in vitro*	2D SDS PAGE Spot detection and quantification	BM and adipose tissue derived cells were very similar and can be interchangeable use for cell therapy.

Within the context of tissue engineering there has been research attempting to test the effects of HA scaffolds on the proteome of different mesenchymal stem cell lines (Table 1.6). The investigations differ in terms of the type of the HA scaffold utilised, the mesenchymal stem cell line used, the methodology of proteomic analysis and/or how cell differentiation was performed. One of the major downfalls of these studies is the lack of an attempt to induce differentiation after cellular attachment on target HA scaffolds. Mesenchymal differentiation would be ideal as it best mimics an *in vivo* scenario where the aim of the scaffold is to allow attachment, proliferation and induce healing through specialisation of cellular lineage. Commercialised cell lines of

osteoblasts are used to compare bone healing in all of the HA studies. The validity of a finding becomes questionable if two cell lines of the same species origin are compared without the host cell line being controlled (e.g individual genetic differences between the same species of cell lines do exist). Therefore it is recommended that experimentation should only occur on primary cell lines as their genetic lineage can be controlled along with their passage number.

Table 1.6: A list of studies utilising proteomic methodologies to investigate the effect of HA on mesenchymal stem cells for bone tissue engineering The cell line investigated on a particular model is shown with the current methodology used. This is just a summary of published seeded HA proteomics articles.

Cell Type	Species	Model	Method of analysis	Reference
Osteoblast	Human	Proteome of nano tubes HA seeded osteoblasts	iTRAQ-coupled 2D LCMS/ MS	[171]
Osteoblast	Human	Proteome of HA nano particles seeded osteoblasts	iTRAQ-coupled 2D LCMS/ MS	[172]
Serum	Rat	Determine proteins selectively adsorbed onto HA	LC/MS/MS	[173]

1.6.3 The employment of ADSC seeded scaffolds for Bone Tissue Engineering.

Bone tissue engineering studies that utilise ADSCs can be categorised as either *in vitro* characterisation studies which investigate osteogenic potential in novel or commercially available scaffolds or *in vivo* studies performed on animal models for bone defects. As previously mentioned, current scaffold materials facilitate the attachment of ADSCs by providing an interconnected pore structure to allow proliferation, differentiation and in some cases formation of blood vessels, however poor mechanical properties such as brittleness is one of the major limitations of 1st generation ceramic scaffolds [174]. Currently, 2nd and 3rd generations of bioabsorbable scaffolds can utilise bioactive materials to gain bioactivity which makes them the preferred scaffolding material for ADSCs in clinical trials.

When considering current studies of ADSC applications for bone tissue regeneration, animal models are common. This is due to the ethical and safety implications coherent in human clinical trials which made alternative forms of research desirable. However, there have been clinical trials that have demonstrated that human ADSCs can be used to reconstruct craniofacial defects in humans, as well as other common areas of bone injuries [175].

As previously mentioned in this review, calcium phosphate ceramics are the most common studied scaffold in bone tissue engineering due to their structural and chemical similarity to bone. The most common type of calcium phosphate ceramic studied is HA, [176-178]. The seeding of human ADSCs on HA has been shown to induce callus vascularisation and mend critical size defects in rats [176] and nude mice [179]. Native HA cannot stimulate or induce osteoinduction to the site of implantation [138]. however most HA scaffolds can be easily fabricated to contain biomolecules which do have the potential to induce such effects [180] .

Numerous rat models utilising ADSC seeded HA has allowed the first clinical study evaluating ADSC seeded scaffolds for human use [175]. Since then, the presence of osteogenesis in bioabsorbable materials loaded with human ADSC has been shown in both *in vitro* [181] and *in vivo [182].* A clinical study has also demonstrated that human ADSC seeded ceramic scaffolds can heal critical-sized defects [183] Some 1st generation bioceramics can promote ADSC attachment, proliferation, vascularisation and osteo-differentiation however they are considered as poor candidates for future bone regeneration scaffolds due to their weak mechanical properties and brittle behaviour which are not ideal for future scaffold-assisted bone regeneration. However, later generations of ADSC seeded calcium phosphate ceramics have demonstrated better mechanical and biological properties thus making it the preferred scaffolding material for future clinical trials [184].

The field of tissue engineering lacks standardisation of applied techniques such as scaffold fabrication, cell passaging, seeding techniques, and the type of media utilised for cell culturing which makes the comparison between different studies difficult.

Furthermore, the fabrication of a plethora of different bioceramics lack guidelines for their proper utilisation for clinical studies as the different properties of the scaffold materials may compromise safety concerns which ultimately burdens the technology as an innovative clinical tool [185,186]. Future clinical trials and potential bone regeneration therapy tools rely on the implementation of regulated standardisation of ADSC research.

All previously mentioned studies, successful or not, did not investigate the biological context of their findings due to a lack of proteomic and genomic approaches. The advantages of ADSC over other mesenchymal stem cell lines has already been stated before in this review and thus research into its implementation as a therapeutic product with HA scaffolds is necessary to determine their applicability, especially with the rising incidence of musculoskeletal injuries. A proteomic characterisation of ADSC seeded biomimetic scaffold would reveal the underlying molecular interactions involved in the osteoconductive and potential osteoinductive properties of HA while also revealing the essential protein biomarkers involved in osteo-differentiation which could be pursued as an alternative form of therapy.

1.7 Topics and Aims in this Thesis

- **AIM ONE:** To characterise the effect of coralline hydroxyapatite (HA) on rat ADSCs for 14 and 30 days and identifying the protein profile using LC/MS/MS and assessing cellular proliferation with scanning electron microscopy (SEM) and the indirect effect of HA on rat ADSCs *in vitro* with histological staining (Chapters II).
- **AIM TWO**: To characterise the insoluble membrane subproteome of seeded rADSCs through ultracentrifugation isolation and subsequent solubilisation (Chapters III).
- **AIM THREE**: To characterise the effect of human adipose derived stem cells (hADSCs) on multiple coralline scaffolds (coralline carbonate, high density HA, nanoporous HA and macroporous HA) by identifying and comparing their proteomic profile with LC/MS/MS and detecting their cytokine and chemokine expression with a Bioplex assay while assessing cellular proliferation with SEM (Chapter IV)

It is hypothesised that during the stem cell and coralline co-culture, osteogenic proteins that modulate mesenchymal stem cell differentiation and proliferation will be expressed, these will be identified in terms presence and absence of protein expression. The findings of this study will further the knowledge on proteomic interactions of stem cells with coralline biomatrices and its surrounding tissue. Initially, the project will aim to investigate and compare the effects of well-established coralline biomaterials on ADSCs in an attempt to uncover the proteomic signature during scaffold induced bone regeneration. The scaffold is produced from hydrothermally converted corals (*Porites*), that uniquely possess the natural architectural similar to bone and composed with natural elements advantageous to the healing process[187,188]. At an applied level, this information will allow for a better appreciation on the role of pore sizes and micro constituents from corals in influencing

the cellular behaviour of stem cells and the pathways in which they differentiate. Additionally, not much is known about the molecular mechanism through which coralline scaffolds induce their osteoconductive effect on their target cells. Multiple *in vivo* studies either report complete bone resorption on the implant site [189] or partial resorption accompanied with inflammation of the target [190]. This thesis aims to report the changes in the ADSC proteome to produce further clarification on the efficacy of coralline materials as an orthopaedic construct.

Chapter Two: (Paper I): The Proteomic Comparison of Hydroxyapatite Seeded Rat Adipose Derived Stem Cells To Investigate Osteogenic Lineage

Declaration

I declare that the following publication included in this thesis in lieu of a chapter meets the following:

- More than 50% of the content in the following publication included in this chapter has been planned, executed and prepared for publication by me
- The work presented here has been prepared for publication and will be submitted
- The initial draft of the work has been written by me and any subsequent changes in response to co-authors and editors reviews was performed by me
- The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis.

Publication title: *The Proteomic Comparison of Hydroxyapatite Seeded Rat Adipose Derived Stem Cells To Investigate Osteogenic Lineage* Authors: Krishneel Singh, Jerran Santos, Benjamin B. A. Raymond, Besim Ben-Nissan, Matthew P Padula , Bruce Milthorpe.

Candidate's contribution (%): above 50 %

Journal name: Journal of Regenerative Medicine Volume/ page numbers:

Status: To be Submitted:

I declare that the publication above meets the requirements to be included in the thesis.

Candidate's name: Candidate's signature: Date:

2.1. Introduction

Musculoskeletal conditions such as osteoporosis, osteoarthritis, rheumatoid arthritis and skeletal injuries affect millions worldwide, making it one of the most common causes of severe chronic pain and physical disability. With increasing and re-emerging prevalence of injury, there is an increase in demand for therapies that can replace damaged tissues or aid in its repair [4]. Recently, augmented biomaterials have emerged as the next generation of advanced materials for bone regenerative therapy [191].

Biomaterials are intended to interface with biological systems to evaluate, treat, augment, or replace any tissue, organ, or function of the body [38]. The nature of these interactions between a given biomaterial and its surrounding cells and tissues can be instructive or inductive in stimulating important biological effects. Inductive biomaterials can induce tissue formation with or without the aid of exogenous growth factors implemented within the material [39], while instructive biomaterials stimulate tissue formation through the host's own regenerative mechanisms [40]. Biomaterials differ greatly in comparison to surgical devices as the latter does not intimately associate with local host cells and tissues for improved healing and repair [192]. Biomaterials gain their advantage where much needed biological actions important in driving tissue regeneration are incorporated within the material as chemical and/or physical cues. These inductive cues have been investigated in an attempt to dissect the interactions between a material and its surrounding environment [42].

Several types of bioactive materials that have clinical relevance have been reported for musculoskeletal tissue engineering in the last few years, including bioactive glasses ($Na_2O–CaO–SiO_2–P_2O_5$) [193], β-tricalcium phosphate (β-TCP) ($Ca_3(PO_4)_2$) [194], β-wollastonite ($CaO\text{-}SiO_2$)[195], A-W (Apatite-Wollastonite) glass ceramics [196] and Silicon nano-plates [197]. In spite of the range of materials available, difficulties persist with many of these materials including a lack of osteoinstructive properties, poor biocompatibility, and insufficient biodegradation [198]. Demineralised bone matrix

(DBM) has been found to be clinically advantageous as it has been shown to help bone regeneration [199], although its recent application has been limited by batch to batch variability, immunogenicity issues, and high production costs [200]. Converted hydroxyapatite (HA) is a type of calcium phosphate material that has shown promise in developing a strong matrix for use in bone regeneration, by being biocompatible, biodegradable and can act as a load bearing surgical device with several osteo-architectural properties and drug delivery applications [201].

Of the different types of calcium phosphate, HA has been heavily investigated with clinical model studies dating back to the 1970's [83]. Porous HA is favoured over dense HA as porous HA allows better integration into the bone. When HA has been placed into bone defects, bone growth into pores has been observed through changes in mechanical measurements, a correlation well established in the field of bone engineering [84].

Although the abovementioned reports have investigated converted HA for a range of applications, the interaction of converted HA with biological tissue to investigate cellular phenotype through proteomics has not yet been performed. In our study, we seed adipose derived stem cells into porous converted HA to test its biocompatibility and osteogenic potential in the absence of any osteoinductive factors such as bone morphogenetic proteins-2 (BMP-2) or dexamethasone. The proteomic characterisation of ADSC seeded HA reveals an underlying molecular interaction involving osteoconductive and osteoinductive pathways. A complementary SEM and Western blot analysis showed essential protein biomarkers involved in osteodifferentiation. This is the first study showing that converted HA can induce osteogenic differentiation of ADSCs. Future applications of seeded converted HA can aid in the urgent need for therapeutics in the field of regenerative medicine.

2.2 Materials and methods

2.2.1 Production and characterization of scaffold

The coralline hydroxyapatite was sourced from the exoskeleton of Porites coral and shaped in the form of cylindrical blocks before being hydrothermally converted and synthetically produced using the protocol by Hu *et al* [201].

2.2.2 Isolation and culture of plastic adherent rat osteoblasts and rADSCs.

Adult *Rattus norvegicus* (Fischer species) were used for the isolation of rat primary bone cells and rat ADSCs (rADSCs). Animals were housed and cared for under standard conditions and were euthanized in a CO_2 chamber after which the inguinal fat pad was harvested and ADSCs were isolated using previously published methods [139]. All subsequent steps were conducted under sterile conditions. Fat pads were rinsed twice in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Australia). The fat pad was then minced with a pair of scissors briefly till a fine slurry was formed, before digestion with collagenase type 1 (Gibco, Australia) for 45 minutes at 37°C. The suspension was then centrifuged at 1600g for 10 minutes at 4^{o}C to separate adipocytes from the stromal vascular fraction (SVF). The SVF pellet was resuspended in 3ml of DMEM and then pipetted carefully on top of 3ml of Ficoll-Paque PLUS (Sigma-Aldrich). Centrifugation at 1600g for 20 minutes removes red blood cells from the SVF.

The ADSCs were then removed from the interface between the Ficoll-Paque PLUS and DMEM. This was then resuspended in 8ml of DMEM to dilute out residual Ficoll-Paque PLUS. The cells were washed twice in DMEM and centrifuged at 1000g. Upon completion of the final wash the pellet is resuspended in basal growth media DMEM Glutmax/F12 (Gibco, Australia) with 10% Fetal bovine Serum (FBS, Invitrogen). The suspension was then aliquoted into 2ml T-25 culture flask and incubated at 37^{o}C at 5% CO_2 for 2 days until ADSCs adhered to the culture flask. Non-adherent cells are eliminated by aspirating floating cells and replacing the media. ADSCs were passaged 3-5 times before utilised in experiments (Santos, 2014, PhD).

Additionally, primary rat osteoblasts were donated by the Santos lab and cultured in basal growth media DMEM Glutmax/F12 (Gibco, Australia) with 10% Fetal bovine Serum until grown to confluency.

2.2.3 Culture of Adipose Derived Stem Cells on Hydroxyapatite

A cylindrical piece of converted hydroxyapatite (dimensions: 5mm length x 3mm diameter) was coated individually by separately placing each scaffold into the 15 mL Falcon tube containing 1mL of the cell suspension (1 x10^6 cells). The tubes were placed in a laboratory centrifuge (Eppendorf Centrifuge 5702) for 1, 2, 3, 4 and 5 minutes at 700 g to ensure efficient seeding density as previously published [202]. Following this the cap of the tubes were released slightly to allow gas exchange and the tubes placed back in a 37°C incubator with 5% CO2. The cells were incubated at 37°C in 5% CO_2 atmospheric pressure and media replaced every 84 hours for a total of 14 days and 30 days when cells were harvested for analysis.

2.2.4 Scanning Electron Microscopy of Cultured Adipose Derived Stem Cells

After 14 days and 30 days of incubation, culture medium was removed and the scaffolds were washed 3 times in PBS. Samples were fixed in 2% glutaraldehyde and 5% formaldehyde in PBS at 4°C overnight. Samples were further washed in PBS and then dehydrated in a graded ethanol series of 20%, 30%, 50%, 70%, 80%, 90%, 96% and two exchanges of 100% ethanol. A 1:1 mixture of absolute ethanol:hexamethyldisilazane (HMDS) was then added followed by two exchanges in 100% HMDS [Sigma-Aldrich], allowing the final volume to dry completely for at least 24 hours. Scaffolds were mounted onto specimen stubs with adhesive carbon tape and silver paint (ProSciTech, Australia) and coated in a ~10 nm layer of carbon using a MED 010 Balzers Union Carbon Coater. Samples were imaged on a Zeiss Supra 55VP using the in-lens detector at 5 kV[203].

2.2.5 Proteomic Characterisation of Seeded and Non-Seeded Adipose Derived Stem Cells

Primary rat osteoblasts, seeded and unseeded rADSCs were grown in ADSC culture

medium for a total of 14 days and 30 days; and harvested with TrypLE Express (Life Technologies, Australia). The collected cells were pelleted after centrifugation at 1000g for 10 minutes. The supernatant was removed and the pellet resuspended in 1% SDS, Triton X-100 solution and 5mM $MgCl_2$ to efficiently lyse cells and obtain all intracellular soluble proteins. The resuspended solution was centrifuged at 16000rcf for 20 minutes at room temperature for the removal of insoluble membrane components. The proteins in the resulting supernatant were assayed to contain 3.17µg/µl of total protein content in a final volume of 77µl. The obtained proteins were reduced and alkylated with 5 mM tributylphosphine and 20 mM acrylamide for 90 minutes at room temperature before being quenched with 1mM dithiothreitol. A total of 100µg of protein was separated by SDS-PAGE and stained initially with Flamingo Fluorescent Gel Stain (Bio-Rad, Australia) followed by visualisation with colloidal Coomassie Blue G-250. The gel lanes were sliced into fourteen equal-sized pieces and then destained using 50% Acetonitrile in 100mM Ammonium Bicarbonate. Proteins were subjected to in-gel trypsin digestion (overnight) followed by LC-MS/MS using an AB Sciex Q-Star Elite quadrupole-TOF instrument [204].

2.2.6 Proteomics MS/MS data analysis

Data obtained from MS/MS spectra were submitted to database search by using the MASCOT search engine (hosted by the Walter and Eliza Hall Institute for Medical Research Systems Biology Mascot Server) against the LudwigNR database (composed of the UniProt, plasmoDB, and Ensembl databases (vQ312. 19 375 804 sequences; 6 797 271 065 residues)) with the following parameter settings: fixed modifications: none; variable modifications: propionamide, oxidized methionine; enzyme: semitrypsin; number of allowed missed cleavages: 3; peptide mass tolerance: 100 ppm; MS/MS mass tolerance: 0.2 Da; charge state: 2+ and 3+, and species restriction of *Rattus norvegicus.*

The resulting MASCOT dat files were transferred to Scaffold software (v4.00.02, Proteome Software, Portland, OR) for further validation and comparison MS/MS-based peptide and protein identifications. Peptide identifications were accepted if their

calculated probability assigned by the Protein Prophet algorithm was >95.0% with at least 2 unique peptides identified. Protein identifications were accepted if their calculated probability was >70.0%. Proteins with only 1 unique peptide identification were manually inspected for quality of spectra.

2.2.7 Histological Staining of rADSCs co-cultured with HA

A small population of rADSCs (1 x 10^4 cells) were coated in a Nunc™ Nunclon™ Vita 6-Well Multidish (Thermo Scientific, USA) with a cylindrical piece of hydroxyapatite placed in the middle of a 6 well plate for 14 days. The cells were subsequently washed in distilled water (MilliQ™) before fixing in 4% paraformaldehyde (pH 7.26) (Sigma Aldrich, USA) for 24 hours followed by further washing in phosphate saline buffer and then stained with standard Haematoxylin and Eosin (H&E) and Alizarin red staining.

2.2.8 Investigating calcium ion release from coralline hydroxyapatite using inductively coupled plasma-mass spectroscopy (ICP-MS)

During *in vitro* culture, hydroxyapatite degrades releasing ionic calcium in to the conditioned culture media, which can be quantified by mass spectrometric analysis. Cylindrical pieces of coralline converted hydroxyapatite (dimensions: 5mm length x 3mm diameter) were immersed in DMEM Glutmax/F12 (Gibco, Australia) and the concentration of calcium ions released into the conditioned medium measured every 72 hours for 30 days. The collected samples were then diluted in 1% nitric acid before ICP-MS analysis.

The ionic compositions of the samples were quantified by ICP-MS using an Agilent Technologies 7500 ce series ICP-MS (Agilent, Sydney, Australia). Sample introduction was made via a micromist concentric nebuliser (Glass expansion) and a Scott type double pass spray chamber cooled to 2 °C. The sample solution and the spray chamber waste were carried with the aid of a peristaltic pump. The ICP operating parameters and the lens conditions were selected to maximise the sensitivity of a mixture of 1% HNO_3, 1% HCl solution containing 1 ng/mL of Li, Co, Y, Ce and Tl. Helium was added into the octopole reaction cell to reduce interference. Calibration curves were

constructed and the results analysed using Agilent Technologies Masshunter software. The data obtained were uploaded into Microsoft Excel to produce a scatter plot. Additional statistical analysis was also performed in Microsoft Excel between the variables with an ANOVA analysis.

2.3 Results

2.3.1 Scanning Electron Microscopy of Cultured Adipose Derived Stem Cells

The purpose of this study was to investigate the proteome changes in rADSCs and the extent of osteogenic influence of converted hydroxyapatite on seeded rADSCs. To evaluate the effectiveness of our seeding methodology, SEM was employed to visualise unseeded and ADSC seeded converted hydroxyapatite samples.

The granularity of the unseeded day 14 HA samples were shown to have an irregular topography and a rough surface (Figure 2.1a). The evaluation of HA seeded with the rADSCs showed a monolayer of cells forming over the 100µm pores after 14 days (Figure 2.1b). Day 30 unseeded HA samples revealed uniform pores of 100µm in diameter (Figure 2.1c) while a large group of aggregated cells on the granular surface is observable on the Day 30 HA seeded samples (Figure 2.1d).

Although the cells had fibroblastic morphology [205], their diameters were approximately 20µm wide, which is similar to plastic adherent rADSCs. The presence of mono-layered cells over pores and granules were consistent throughout the imaged SEM seeded HA.

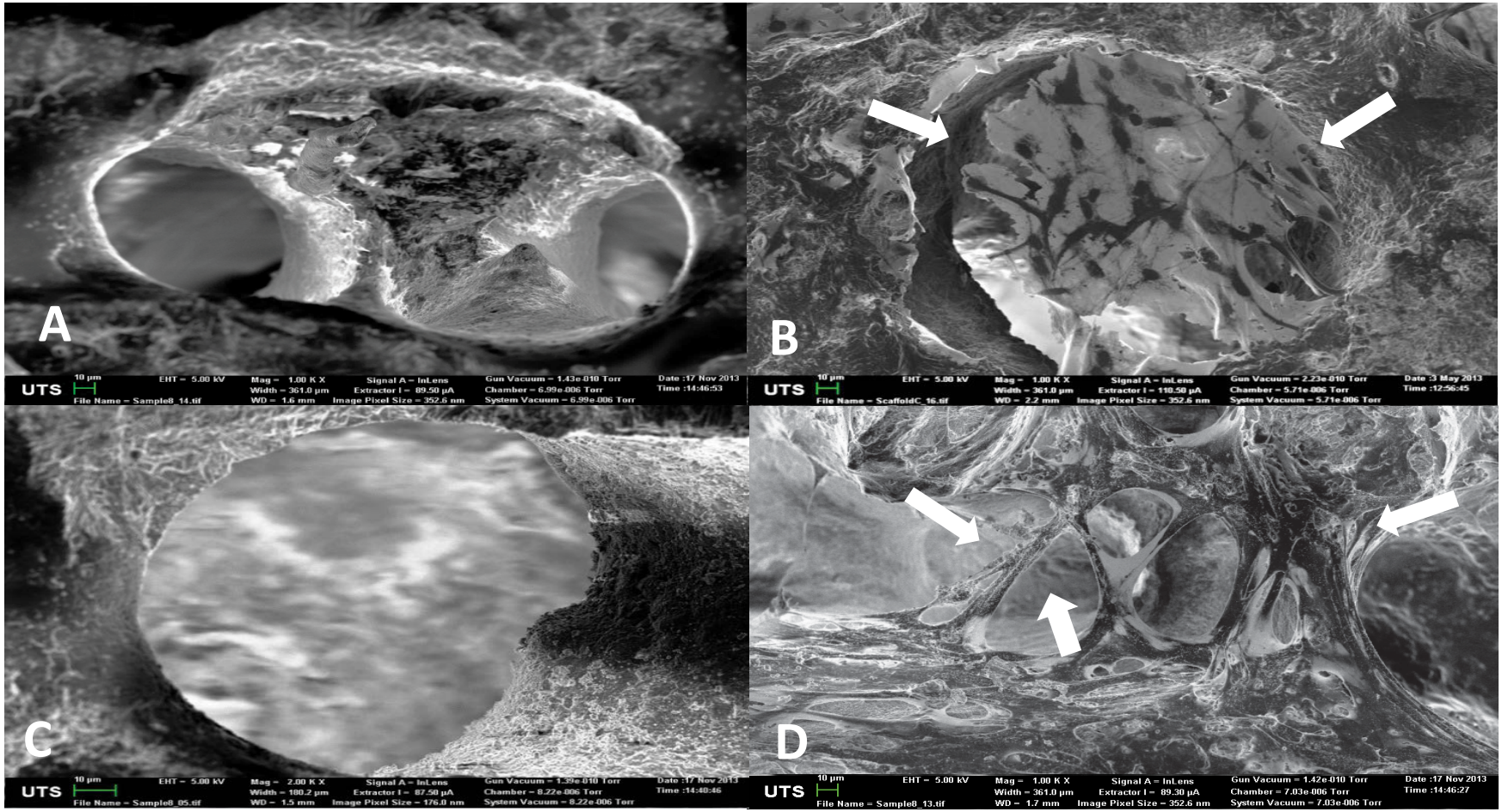

Figure 2.1 Scanning electron micrographs (SEM) showing the surface structure of cross-sections of the HA. (A) Unseeded for 14 days; (B) 14 Days Seeded showing a monolayer of cells proliferating across the porous HA (white arrows) (C) Unseeded for 30 days (D) 30 Days seeded showing a high abundance of cells proliferating across the interconnected pores of HA (white arrows).

2.3.2 Proteomic Comparison of Primary Osteoblasts, Seeded and Non-Seeded ADSCs

The study aimed to investigate a change in the rADSC proteome signature after 14 and 30 days of initial HA seeding as other studies have shown that both human and rat mesenchymal stem cells begin to deposit calcium phosphate mineral within the extracellular matrix, and express osteogenic genes and proteins after 2 to 4 weeks *in vitro* [206,207].

The data from the LC/MS/MS analysis identified a total of 570, 228 and 276 proteins for basal rADSCs, Seeded HA Day14, and Seeded HA Day30 respectively. All proteins were identified with at least two sequenced peptides and a FDR parameter of <5%. Functional classification of the proteins according to the Gene Ontology database and literature surveys revealed that of the 75 unique proteins identified in the Day 30 HA seeded rADSCs cohort , 41 proteins were found to be associated with bone morphogenesis (Table 2.1 and Figure 2.2). These proteins either played a role in calcium induced extracellular matrix deposition (Fibulin-1, Protein Lamc1, Perlecan and Matrix metalloproteinase-14), are involved in the formation of a soft tissue hematoma (Antithrombin-III, Fibroblast Growth Factor 16, Alpha-2 antiplasmin, Dysferlin and Decorin) or are positive regulators of bone morphogenesis (Col2a1 Collagen alpha-1(II) chain, Transforming growth factor beta-1, Biglycan, and Insulin-like growth factor 2 receptor). Interestingly, there was an increase in the number of unique proteins identified from 26 to 75 between Seeded Day14 and Day30 cohorts respectively.

Additionally, GO annotation of the 126 proteins shared between the three datasets revealed that a large percentage of these proteins play a role in metabolism (22%), biosynthesis (6%) and organelle development (5%) (Figure 2.3). Specifically, Galectin 1, Profilin 1, Alpha-actinin-1, Cofilin-1 and filamin were proteins annotated to be involved in metabolism and subsequently found to be classified as actin cytoskeletal proteins associated with cellular adhesion.

Further analysis of proteins involved in biosynthesis and development also revealed another set of 26 proteins involved in cell signalling and maintenance such as TGF β–

receptor, TNF-receptor 6 which are readily expressed by mesenchymal cells as downstream regulators of cellular proliferation. Additionally, our data revealed proteins associated with cellular attachment within the day 14 and day 30 seeded HA cohorts (Table 2.2). In our analysis we detected 32 defined proteins involved in regulating calcium ion intake and the presence of four proteins from the Annexin super family (Annexin A3, A4, A7and A11) indicated a response to bone formation as Annexins are abundant in bone matrix vesicles, and are speculated to play a role in Ca^{2+} entry into vesicles during bone morphogenesis [208].

We compared the protein profile of Seeded HA Day 30 proteins with the proteomic dataset of primary rat osteoblasts to understand which subset of seeded HA Day 30 proteins were associated with bone formation (Figure 2.4). This comparison was made against basal ADSCs as a control. Over all, we identified 244 rat primary osteoblast proteins and as previously reported, 570 basal rADSC and 276 seeded HA proteins. The comparison highlighted 31 proteins to be shared between rat primary osteoblasts and seeded HA D30 proteins. This suggests that the phenotype of seeded HA D30 proteins shares only an 11% proteomic similarity to primary osteoblasts. A literature survey of these shared proteins between rat primary bone and seeded HA Day 30 was tabulated (Table 2.3).

In brief, only two proteins were found to play a role in bone formation (Fibronectin, and Actin Related Protein 2/3 complex) while the other proteins of interest were either involved in energy metabolism (Citrate synthase and Electron transfer flavoprotein subunit beta) or the regulation of a free radicals in an highly oxidative microenvironment (Ab2-162, Chloride intracellular channel protein 1 and Thioredoxin-dependent peroxide reductase).

Table 2.1. Listed are some of the proteins identified in the Seeded coralline HA proteome with their uniprot accession number, protein name, protein coverage identified (%) via MS and respective molecular function.

Accession	Protein Name	Coverage	Function
Q6P686	Ostf1 Osteoclast-stimulating factor 1	6%	Induces bone resorption, acting probably through a signalling cascade which results in the secretion of factor(s) enhancing osteoclast formation and activity [209].
Q07936	Annexin A2	4%	Annexins are abundant bone matrix vesicle proteins that have been associated to play role in Ca^{2+} entry into the vesicles and in the formation of hydroxyapatite [210].
F1LRM7	Col2a1 Collagen alpha-1(II) chain	12%	Plays a major role in endochondral bone morphogenesis and endochondral ossification through cellular response from a bone morphogenic protein-2 (BMP-2) stimulus [211]
D3ZCF5	Smurf1 E3 ubiquitin-protein ligase	8%	Involved in the transforming growth factor beta receptor signalling pathway and Runx2 pathway for osteoblast differentiation[212]

D3ZDW8	transforming growth factor beta 1 induced transcript 1	4%	Transforming growth factor-β (TGF-β) plays an important role in bone metabolism by functioning as a coupling factor that links bone resorption and migration of bone mesenchymal stem cells to the remodelling area[213].
Q01129	Decorin	11%	A proteoglycan that has been shown to influence cutaneous wound healing by regulating tissue tensile strength and cellular phenotype [214].
D4A6X1	Dysferlin	6%	Involved in the repair of skeletal muscle tissue through a calcium dependant interaction with Annexin A1 and Annexin A2 during wound healing [215].
Q80ZA3	Alpha-2 antiplasmin	3%	A serine protease inhibitor that inactivates plasmin, which is a key regulator of fibrinolysis and its subsequent blood coagulation pathways [216]
O54769	Fibroblast growth factor 16	6%	Fibroblast growth factor play a role in angiogenesis, wound healing and have shown to induce bone formation by stimulating proliferation and differentiation of osteoprogenitor cells [217]
P23097	Matrix metalloproteinase-13 (MMP-13)	11%	Proteins of the matrix metalloproteinase (MMP-13) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as tissue remodeling and bone repair. [218]
B1WC21	Fibulin-1	27%	Important for the developmental processes of ECM architecture, in particular to structures that heavily play a role in haemostasis and thrombosis such as a haematoma which allows the binding of fibrinogen to induce clots and vascularisation during wound healing [219].

P47853	Biglycan	3%	Biglycan, a small leucine-rich proteoglycan, is a non-fibrillar extracellular matrix component with functions that include the positive regulation of bone formation[220].
Q4FZT0	Stomatin-like protein 2	7%	Works with BMP.to play a role in calcium homeostasis through negative regulation of calcium efflux from the mitochondria [221].
Q64537	Calpain small subunit 1	10%	Calpain small subunit-1 has shown to be essential for osteoblast proliferation and differentiation during osteogenesis [222].
G5BX82	Interleukin-17 receptor (Il-17)	4%	A receptor for the pro-inflammatory cytokine Il-17 which mediates its immunomodulatory effect by initially being induced by IL-23. It has shown to play a role in joint inflammation during arthritis [223].
D3ZZ22	Basonuclin 2	17%	Animal models have shown that a lack basonuclin 2 can lead to lethality from abnormalities of the craniofacial bones. This is because basonuclin 2 is essential for the formation of craniofacial bones by mesenchymal cells during embryogenesis [224].
F1MAA7	Protein Lamc1	6%	Laminins are a family of ECM glycoproteins that constituent a large portion of basement membranes. They have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signalling and outgrowth [225]

O08591	Perlecan	22%	Perlecan is a key component of the vascular ECM where it interacts with a variety of other matrix components to maintain the endothelial barrier. It also aids in vascular homeostasis through its inhibitory role on smooth muscle cells [226]
P47973	Tristetraprolin	14%	Tristetraprolin is a phosphoprotein that regulates the expression of TNF-α by binding to TNF-α mRNA and inducing its physiological degradation to decrease the production of TNF-α during pro inflammatory events [227].
Q10739	Matrix metalloproteinase-14	3%	Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as tissue remodelling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive phosphoproteins which are activated when cleaved by extracellular proteinases [228].
D4AE96	Importin 7	3%	Importain-7 mediates the nuclear import of H2A, H2B, H3 and H4 histones[229].
D4A6X1	Dysferlin	12%	Key calcium ion sensor involved in the Ca^{2+}-triggered synaptic vesicle-plasma membrane fusion. Plays a role in the sarcolemma repair mechanism of skeletal muscle that permits rapid resealing of membranes disrupted by mechanical stress [230].
G3V824	Insulin-like growth factor 2 receptor	4%	Insulin-like growth factor 2 receptor binds the hormone Insulin-like growth factor 2 to mediate its cellular growth regulating effects during cellular differentiation. It has been associated as an important hormone for regulating bone morphogenesis [231]. [232].

Q5M7T5	Antithrombin-III	2%	Antithrombin III inhibits the downstream coagulation pathway of blood clotting through the inactivation of the essential coagulation protein thrombin [233]. Mesenchymal stem have procoagulant activity so the presence of anti-thrombin sets a precedent that hydroxyapatite induces a shift in the functionality of ADSCs, possibly, towards a coagulant pathway. This process actively plays a role in tissue repair during the initial formation of a hematoma.

Table 2.2: Listed are some of the proteins identified to be shared between Day 14 and Day 30 Seeded rADSCs cohorts. The literature survey shows that the identified proteins are involved in adhesion to extracellular matrix proteins, attachment to the HA and proliferation.

Accession	Protein name	Coverage	Function
P70560	Collagen alpha-1(XII chain (Col6a3)	14%	Col6a3 has been shown to bind to ECM proteins and interact with type VI collagen in organizing extracellular matrix components[234].
D3ZFH5	Protein Itih2	6%	Functionally dependent on calcium ions and is involved in post translational modification requiring vitamin K[235].
Q3KR94	Vitronectin	3%	An abundant glycoprotein found in bone [236], vitronectin promotes cell adhesion and proliferation while it inhibits the membrane-damaging effect of the terminal cytolytic complement pathway by promoting several serine protease inhibitors [237].
P22314	Ubiquitin-activating enzyme E1	2%	Part of the super family of E1 enzymes which catalyse the first step in the ubiquitination reactions for processes such adhesion, immune responses and cell division. It is known to interact with ZSWIM 4 for these mechanisms[238].
Q5U362	Annexin A4	8%	Annexin IV belongs to the annexin family of calcium-dependent phospholipid binding proteins. Annexin A4 has *in vitro* anticoagulant activity and also inhibits phospholipase A2 activity from activating the bradykinin pathway [239],
O88752	Epsilon 1 globin	16%	Molecular functions include iron binding and oxygen binding and also involved in platelet production.

Table 2.3. Listed are some of the proteins identified to be shared between rat primary osteoblasts and the Seeded HA Day 30 rADSCs with their respective uniprot accession number, protein name, protein coverage identified (%) via MS and molecular function .

Acession	Protein name	Coverage	Function
P04937-3	Isoform 3 of Fibronectin	6%	Essential for osteoblast mineralisation, fibronectin and all its associated isoforms are involved in the compaction osteoblast compaction through cell mediated fibrillogenesis in the ECM of skeletal bone. Additionally, fibronectin participates in the modulation of type I collagen secretion by osteoblasts during osteogenesis. Additionally, fibronectin has shown promote osteoconductive properties in conjunction with vimentin on a human osteoblast cell line[240].
Q68FP1	Gelsolin	7%	Gelsolin is a protein that aids in ciliogenesis and calcium-regulated formation of filaments [241]
G3V887	V-type proton ATPase subunit a	2%	V-type proton ATPase is induced during osteoclast differentiation and is generally localised in the plasma membrane of osteoblasts. Studies indicate that ATP synthase is a component of the plasma membrane V-ATPase and is essential for bone resorption [242].
Q6MG61	Chloride intracellular channel protein 1	16%	Involved in the regulation of the cell cycle by inserting into the membranes of cells and forming chloride ion channels that are dependent upon pH of the microenvironment. Membrane insertion is also redox-regulated and occurs only under oxidizing conditions [243].
Q8CFN2	Cell division control protein 42 homolog	1%	Maintains and extends the filopodia of migrating cells. Filopodia are the formation of thin, actin-rich surface projections involved in the chemotropic locomotion of infiltrative cells [244].

P61983	14-3-3 protein gamma	9%	A type of adapter protein which is implicated in the regulation of a large spectrum of both general and specialised signaling pathways. This interaction results in the regulation of most downstream signaling pathways for differentiation and cell division [245].
B2GV73	Actin related protein 2/3 complex, subunit 3	12%	Actin rings are vital for osteoclastic bone resorption, and actin-related protein complexes are important regulators of actin polymerisation. Short knockdowns of the protein complex disrupts the osteoblast and osteoclast coupling [246].
Q68FU3	Electron transfer flavoprotein subunit beta	5%	Important in the mitochondrial process of AMP production during high metabolic activities of the cell cycle.[247]
Q7TPJ0	Translocon-associated protein subunit alpha (TRAP)	3%	TRAP proteins are part of a complex whose function is to bind calcium to the ER membrane and thereby regulate the retention of ER resident proteins [248].
Q9Z0V6	Thioredoxin-dependent peroxide reductase, mitochondrial	3%	Interacts with other oxidoreductase enzymes in scavenging the microenvironment for free radicals. This process allows inhibits oxidative stress during elevated biological processes of cells [249].

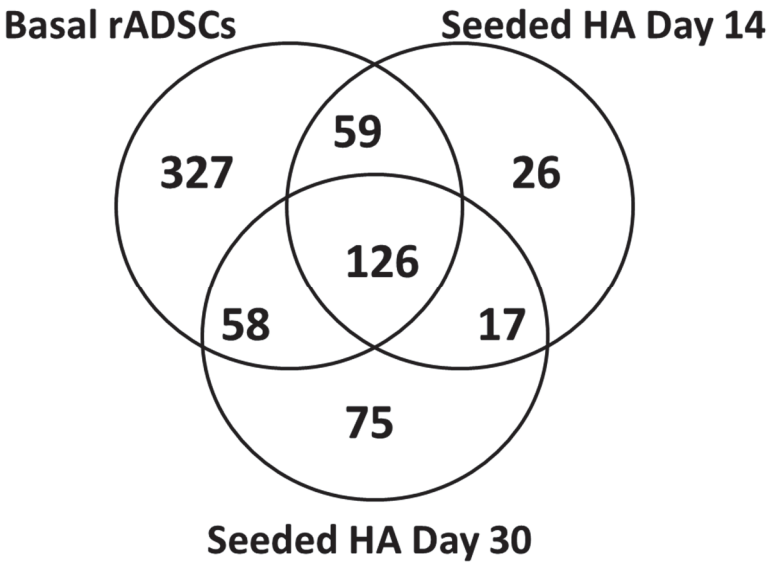

Figure 2.2: Venn diagram of proteins identified between HA seeded ADSCs for 14 days (Seeded HA Day 14), 30 days (Seeded HA Day 30) and basal rADSCs (Basal rADSC) *in vitro*. In total, 570 basal rADSCs proteins were identified while 75 proteins were uniquely identified in day 30 seeded ADSCs. Of the 75 proteins, <50 proteins were found to play a role in bone morphogenesis.

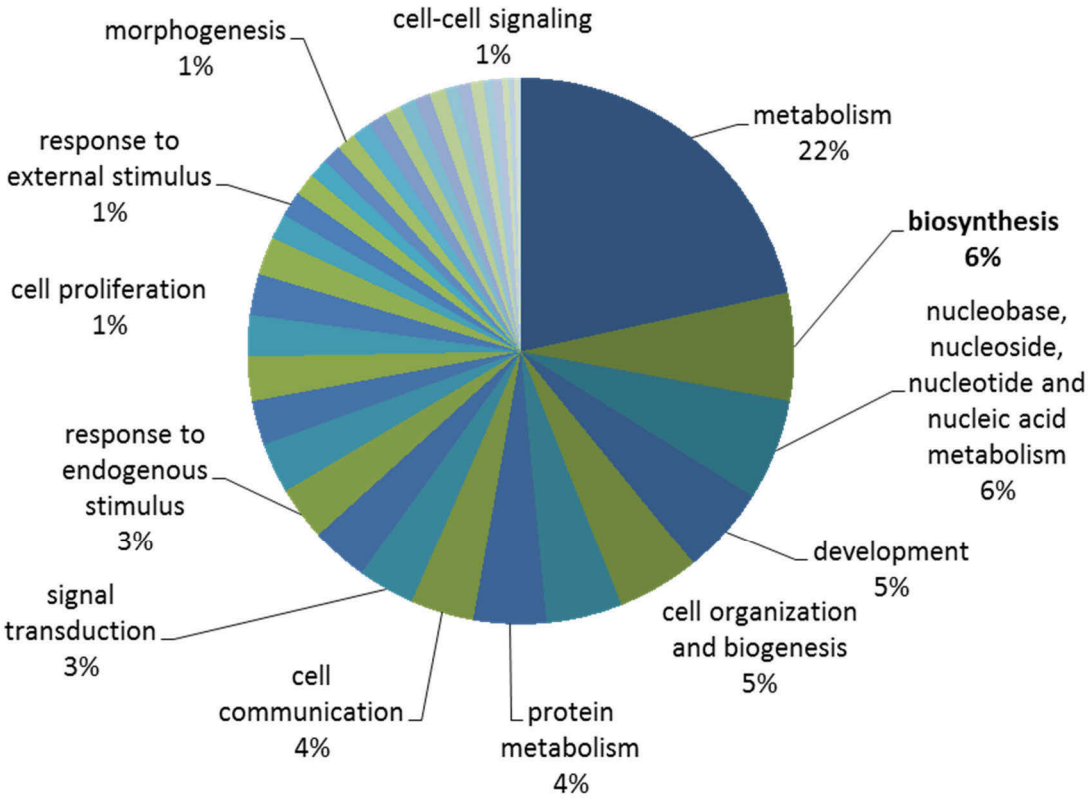

Figure 2.3: The GO annotation of the proteins shared between the three ADSC cohorts (Seeded HA Day 14, Seeded HA D30 and basal rADSC). A large percentage of proteins have been annotated to play a role in metabolism (22%), biosynthesis (6%), development (5%), cell organisation and biogenesis (5%). The other biological functions of these proteins also include protein metabolism, cell communication and signal transduction

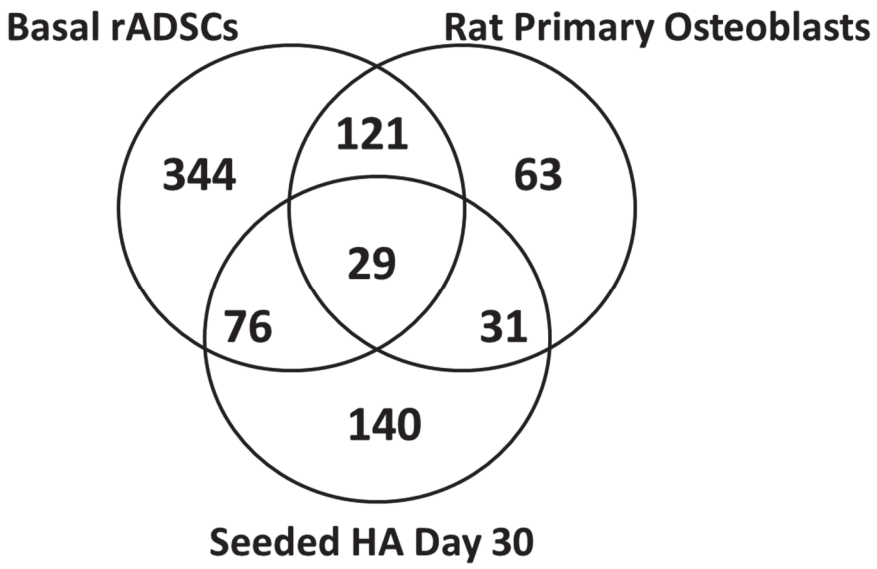

Figure 2.4: Venn diagram of proteins identified between seeded HA ADSCs for 30 days (Seeded HA Day 30), isolated primary osteoblasts (Rat Primary Osteoblasts) and basal ADSCs (Basal rADSCs)**.** A total of 140 proteins were uniquely expressed in HA D30 cohort while 31 proteins were shared between the primary bone cells and HA seeded ADSCs. Furthermore, 29 proteins were shared between all three cohorts.

2.3.3 Histological staining of seeded rADSCs and *in vitro* evaluation of coralline hydroxyapatite degradation.

Histological staining was performed to evaluate presence of calcium deposition in rADSCs co-incubated with HA over 14 days. Our results showed that rADSCs cultured in the absence of HA does not stain positive for divalent ions (Figure 2.5a) while in comparison rADSCs co-incubated with HA did stain positive for divalent ions such as calcium (Figure 2.5b). The presence of calcium nodules seen throughout the images is similar to what is noted in the literature for signs of osteogenic differentiation[250]. This suggests that the direct attachment of rADSCs to the HA is not be required for inducing an osteoblast like phenotype seen within our results. However, it is possible that both the ion release and direct contact with the scaffold may work in tandem to induce an osteogenic effect on rADSCs.

The dissolution of calcium from coralline hydroxyapatite was assessed using ICP/MS to quantify the release of calcium ions *in vivo* during pre-determined time intervals (0-30 days) (Figure 2.6).This allows us to investigate if a biologically relevant concentration of calcium is released in the microenvironment of coralline hydroxyapatite (Figure 2.6). The results show that over time, the amount of calcium ions released is increased with a peak concentration of 197 ppm after 30 days and an initial concentration of 154 ppm on day 3. The amount of calcium ion released from coralline hydroxyapatite corroborates with our detection of divalent cations in the previously mentioned histological staining study.

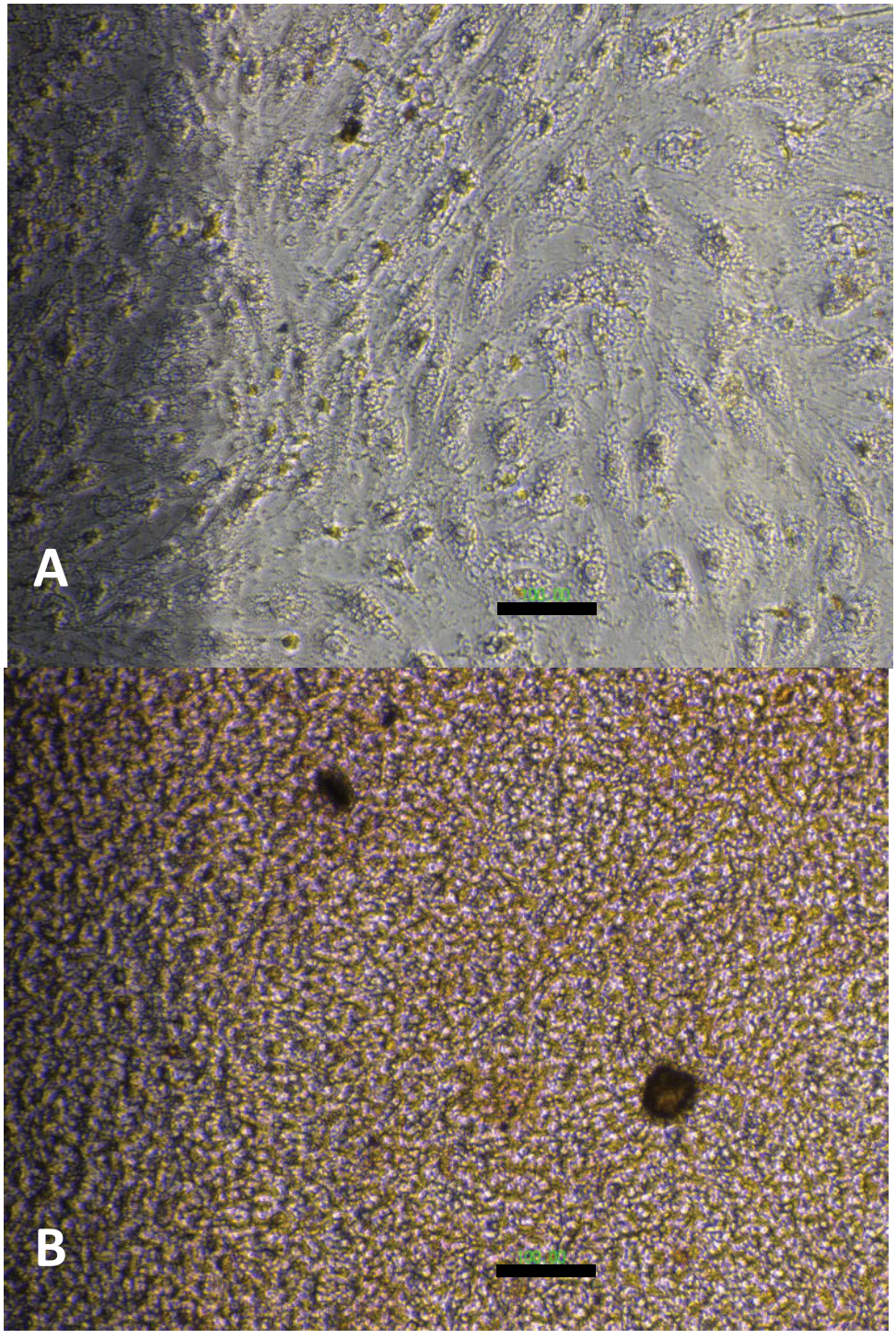

Figure 2.5. Micrographs of osteogenic assessment of rADSCs *in vitro* (A) in control culture medium for 30 days and (B) HA and control medium for 30 days. Large calcium deposits can be seen stained positive in image B however, image only shows basal ADSCs which remained unstained by Alzarin Red. The scale bar represents 100µm (black bar)

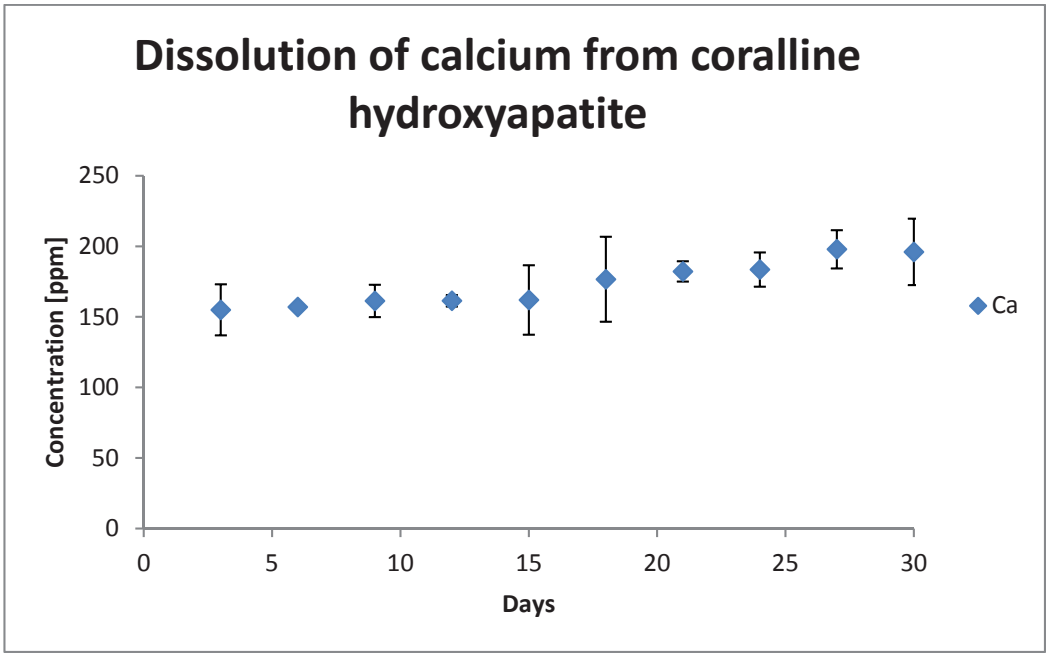

Figure 2.6: Release of calcium (Ca^{2+}) from converted coralline hydroxyapatite in ADSC medium at allocated time points. The results indicated a statistically significant increase in calcium concentration between day 3 and day 30 (p=0.041)

2.4 Discussion

Within this study, converted coralline hydroxyapatite was seeded with rat adipose derived stem cells for 14 and 30 days and then subsequently, had their biological response for osteogenic change evaluated through tandem mass spectrometry, light microscopy and scanning electron microscopy. Initially, we evaluated the compatibility of rADSCs seeded onto coralline HA and observed a monolayer of cells proliferating across the porous architecture of HA after 14 and 30 days. This suggests that our seeding protocol can be applied to coating coralline HA that has a pore interconnectivity of 100 μm - 150 μm [251]. Prior studies that have investigated the attachment of cellular material onto orthopaedic constructs have shown that pore size plays a role In seeding efficiency [252]. Kitagawa *et al* showed that adherent efficiency was greater in scaffolds with a 30 μm pore interconnectivity than in comparison to scaffolds with 70μm pore interconnectivity [253]. To be able to coat coralline HA with a large pore size, other studies have employed additional surface protein coating with either fibronectin or laminin to enhance cellular adherence. [254,255]. Therefore our study may be the first to utilise a different seeding protocol to coat ADSCs onto coralline HA.

Our LC/MS/MS analysis of seeded HA rADSCs identified the expression of osteogenic proteins in the absence of external growth factors such as dexamethasone and TFG-β, which are commonly utilised to promote osteogenesis in mesenchymal stem cells [139] and in similar coralline HA constructs [256].This indicates that the expression of these osteogenic proteins may be influenced by the surface chemistry of the coralline HA as the conditioned medium played no role in directing the rADSCs towards an osteogenic phenotype.

Interestingly, a wider array of osteogenic proteins was expressed after shifting the length of seeded HA culture incubation from 14 days to 30 days. This increase in the number of osteogenic proteins identified included osteoclast stimulating factor 1 and interleukin-17; both of which synergistically interact with one another in regulating the

RANKL/TNFα pathway of bone morphogenesis [257]. Furthermore, the presence of biglycan, decorin and tristetraprolin has been shown to promote angiogenesis by immature bone marrow mesenchymal stem cells as both decorin and biglycans are known multifunctional proteoglycans that induce the akt/protein kinase pathway in regulating tissue repair *in vivo* [258]. The akt/protein kinase signalling transduction allows the formation of capillary-like structures while also decreasing the apoptosis of regenerative fibroblasts *in vitro* [259]. Therefore, the presence of the aforementioned proteins are important for directing ADSCs towards a tissue regenerative pathway and regulating the initial stages of mineralised bone growth [260] while stomatin-like protein 2 and decorin have been shown to be commonly expressed during the later stages of bone tissue repair as well [261,262].

Many studies have attributed this osteoconductive property of HA to its high pore interconnectivity which allows attachment and proliferation of osteoprogenitors cells to the biomaterial[20]. This was also evident in our proteomic analysis through the expression of extracellular matrix (ECM) adhesion proteins. Interestingly, these proteins of interest were found to be shared between Day 14 and Day 30 seeded HA cohorts and were identified to be collagen alpha-3(VI) chain (Col6a3), vitronectin and annexin A4.

Col6a3 has been shown to bind to ECM proteins and interact with type VI collagen to mediate organisation of glycoproteins in the extracellular matrix [234,263,264]. Abundant glycoproteins such as vitronectin, which promotes cell adhesion and proliferation while inhibiting the membrane-damaging effect of terminal catalytic complement pathways through the promotion of several serine protease inhibitors [237].

A plausible explanation for the presence of calcium regulatory proteins such as (dysferlin and stomatin-like protein 2) within this study could be due to the release of calcium from the HA scaffold into the cell culture medium or the microenvironment. As shown in our microscopy results and ICP/MS analysis; the former showed the presence

of divalent cations such as calcium deposits present throughout the image (Figure 2.5b) and the latter correlated with an increase of calcium ions being released from the scaffold over time (Figure 2.6). These results agree with a similar study performed by McCullen et al [265] who demonstrated that human adipose derived stem cells (hADSCs) undergo a similar phenotypic change with the formation of calcium nodules throughout the cells. A subsequent X-ray diffraction study by the same authors characterised the mineralised nodule to have a crystalline structure characteristic of hydroxyapatite[265]. Interestingly, the presence of the aforementioned ions is key catalyst for a large number of enzymes identified in our proteomic analysis of the rat primary bone cells and the Day 30 Seeded ADSC dataset. Enzymes such as isocitrate dehydrogenase which provides positive feedback by ATP during high metabolic activity, such as differentiation and proliferation [266] are known to be regulated by the presence of calcium [267], which is abundant in the HA microenvironment [268].

Additionally, the study by Charkravorty *et al* showed that the rough surface topography of biomaterials activates the TGF-β/BMP and non-canonical WNT/Ca^{2+} signalling pathway for osteogenic differentiation in mesenchymal stem cells [269]. This further suggests that the microenvironment of the HA may be playing a role in the osteogenesis observed within our study. However, the HA scaffold utilised within this study is uniformly interconnected with pores which limit the interaction of seeded ADSCs with the rough surface topography required. This implies that not all cell populations within the scaffold will be affected by the osteoconductuve factor of HA since a subpopulation of cells did continue to proliferate across the uniform pore sizes as evident in our SEM results (Figure 2.1C and 2.1D). Therefore, we speculate that the majority of osteoconductive properties influencing the osteodifferentiation of rADSCs in our study were due to the release of calcium ions from the HA. This is evident by other genomic and transcriptomic studies which demonstrated that an increase in HA dissolution by rat bone marrow stromal cells upregulated the expression of the osteogenic transcriptional factor Runx2 and the osteogenic genes, fibroblast growth factor-2 and transforming growth factor-β1 [270].

To our knowledge, this is the first study to identify the temporal changes in protein expression profiles of rADSCs seeded onto converted coralline HA and to show the expression of osteogenic associated proteins after 14 days and 4-days of initial seeding. Other studies have alternatively examined gene expression in mesenchymal stem cells [271] and osteoblasts [272-274] seeded on hydroxyapatite. These studies have shown that there is an increase in expression of osteogenic genes such as runx2, Bmp-4 and Col2 after seeding with the aforementioned cell types. It is important to note that gene expression is only an indirect method for assessing the relative abundance of protein expression.

Furthermore, this is the first application of proteomics to identify a broad protein profile of seeded rADSCs. The most noticeable observation from our LC/MS/MS analysis is the large number of proteins identified in our basal rADSC dataset. The great number of proteins expressed by rADSCs suggests that the multi-potency of the stem cells plays an influential role on the expression of a diverse array of proteins, due to a lack of phenotypic commitment. The “narrowing down” of protein count from basal rADSCs to HA treated rADSCs may suggest an alternate rADSC commitment towards a defined phenotype. This became evident in our proteomic comparison of rat primary osteoblasts and seeded HA. Within this comparison only 31 proteins were found to be shared between seeded and rat primary osteoblast cohort. Therefore, the large number of basal rADSCs identified may be attributed to the lack of phenotypic commitment seen within the basal stem cells and instead, large repertoires of proteins are expressed to maintain their multi-potency and self-renewal capacity.

Interestingly, the presence of well-established osteogenic proteins such as osteopontin and osteocalcin [119,168] were only identified in the protein profile of rat primary osteoblasts . However the expression of other potential osteogenic proteins such as the family of Annexins (A1, A2 and A3), Vat1 Synaptic vesicle membrane protein and filamin C suggests that complete osteogenic differentiation could be accomplished if we allowed the seeded HA to be incubated beyond 30 days. This suggests that the proteins identified in seeded rADSCs may be involved in pre-osteogenic differentiation.

2.5 Conclusion

Coralline hydroxyapatite (cHA) allows cellular attachment and proliferation with growing evidence demonstrating that the biomaterial is also osteoconductive. Progenitor cells seeded onto cHA have shown to differentiate into bone like cells yet the proteins involved in mediating this effect has not been established. Here we show that rADSCs seeded onto HA for 14 and 30 days begin to express proteins that implicate a phenotype similar to osteogenic cells. The proteins expressed by rADSCs increased when the progenitor cells were allowed to be treated with cHA for an additional 16 days. Interestingly, a proteomic comparison of seeded HA and primary rat osteoblasts share some similarity however the presence of osteo-regulatory proteins suggest that the cells aren't mature osteoblasts and instead they may possibly be at intermittent stage of differentiation. The expression of calcium binding proteins does also suggest the rADSCs are responding to a source of calcium from their hospitable microenvironment inside the coralline matrix.

Here we show that direct contact with cHA may not be the only factor involved at inducing a phenotypic change in the rADSCs. Co-incubation of cHA in the same culture medium as rADSCs allowed the formation of calcium nodules, a common morphological trait observed in histological studies of chemically induced osteo-differentiated mesenchymal stem cells.

Here we also show the growth and proliferation of rADSCs within the coralline matrix of HA after 14 days where we observed the formation a monolayer of cells across the pores of cHA; and after 30 days, the growth of ADSCs across the interconnected pores of cHA. Previous studies have not utilised LC/MS/MS to define the protein expression profiles of rADSCs seeded onto cHA. Therefore, this study offers a different biological perspective of the interaction between coralline matrix and seeded ADSCs that allow us assess the potential of ADSC coated coralline hydroxyapatite as an orthopaedic construct for bone tissue engineering.

Chapter Three: (Paper II): The Membrane Proteomic Comparison of Hydroxyapatite Seeded Rat Adipose Derived Stem Cells To Investigate Osteogenic Lineage

Declaration

I declare that the following publication included in this thesis in lieu of a chapter meets the following:

- More than 50% of the content in the following publication included in this chapter has been planned, executed and prepared for publication by me
- The work presented here has been prepared for publication and will be submitted

- The initial draft of the work has been written by me and any subsequent changes in response to co-authors and editors reviews was performed by me
- The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis.

Publication title: *The Membrane Proteomic Comparison of Hydroxyapatite Seeded Rat Adipose Derived Stem Cells To Investigate Osteogenic Lineage*
Authors: Krishneel Singh, Jerran Santos, Benjamin B. A. Raymond, Besim Ben-Nissan, Matthew P Padula , Bruce Milthorpe.

Candidate's contribution (%): above 50 %

Journal name: Journal of Regenerative Medicine Volume/ page numbers:

Status: To be Submitted:

I declare that the publication above meets the requirements to be included in the thesis.

Candidate's name: Candidate's signature: Date:

3.1 Introduction

Novel orthopaedic constructs hold great potential for the treatment of musculoskeletal conditions, several hundred of which are already under active clinical trials [275]. In particular, the use of calcium phosphate ceramics as substitutes for bone grafts is steadily increasing due to ongoing improvements in its fabrication and utilisation [276]. The most widely used calcium phosphate ceramics are porous hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP), and mixtures of these two that are known as biphasic calcium phosphate (BCP) ceramics.

Mesenchymal stem cells (MSCs) have great appeal for orthopaedic constructs as MSCs combined with HA ceramics have shown to induce bone formation [277]. Additionally, HA seeded MSCs can be used to repair, regenerate and assist in the structural integrity of compromised bones [278]. The primary source of MSCs is the bone marrow however, they can also be isolated from umbilical cord blood, dermis, and the placenta yet, the issue of ethics and their availability impede their applicability in bone tissue engineering [279]. Over the past several years, an alternative source of MSCs found in adipose tissue, called adipose derived stem cells (ADSCs), has garnered large therapeutic interest as they are an abundant source of multipotent stem cells and are relatively inexpensive to acquire.

Additionally, ADSCs have been shown to undergo osteogenic differentiation under varying conditions, including chemical stimulation via the TGF-β pathway [139], gold nanoparticle stimulation via the Wnt/β catenin pathway [280], and physical induction through surface contact with orthopaedic biomaterials [281]. Current osteo-differentiation attempts have been pursued through cellular seeding of ADSCs onto HA and β-TCP scaffolds [282,283]. However, the role that calcium phosphate plays in the induction of osteogenesis on target cells such as ADSCs are poorly characterised. Studies have attributed cell-to-cell communication and interactions to be vital processes for cellular differentiation. Most of these roles take place within the plasma membrane, which facilitate the transfer of biomolecules and chemical signals from the

extracellular environment to the cell. As a result, targeting of membrane bound molecules for drug developments constitutes approximately 70% of human pharmaceuticals available in the current market [284]. Furthermore, it has been suggested that components of the ECM could influence the phenotype of mesenchymal cells [285] and the molecular and biochemical signals by which MSCs interact with their surrounding environment require further elucidation to develop improved scaffolding materials. Therefore, an investigation of the ADSC membrane proteome is necessary as it allows a better understanding of the interactions between ADSCs and their ideal scaffold targets.

Previously, we investigated the effect of coralline HA on rat adipose derived stem cells (rADSCs) and revealed a change in the rADSC proteome after 14 and 30 days (Chapter 2). We identified the presence of several osteogenic proteins after seeding rADSCs onto HA and found that rADSCs under the direct influence of HA begin to adopt an osteoblast-like phenotype. However, the standard shotgun proteomic tool we utilised to investigate the seeded rADSC HA proteome underrepresents membrane associated proteins. This is due to our shotgun proteomic approach having a strong preference for hydrophilic non-membrane proteins as we do not investigate the 'insoluble' fraction obtained during our proteomic analysis.

Therefore, within this study our aim was to investigate the subproteome of rADSC seeded onto HA utilising surface labelling with LC-biotin, trypsin digestion of surface accessible proteins and ultracentrifugation and subsequent solubilisation of membrane proteins. Initially, we compared these techniques and found ultracentrifugation isolation to be the most suitable approach for analysing the subproteome of seeded HA, due to its wider coverage of proteins identified.
Finally, we compared the subproteome profile of seeded rADSCs with primary rat osteoblasts and basal rADSCs and identified a number of cellular adhesion molecules, CD markers and more importantly, the expression of ECM molecules involved in osteo-regulation which includes fibronectin, calnexin, calreticulin and endoplasmin.

3.2 Materials and Methods.

3.2.1 Isolation and culture of plastic adherent rADSCs and rat primary osteoblasts.

Adult *Rattus norvegicus* (Fischer species) were used for the isolation of rat primary bone cells and rat ADSCs (rADSCs). Animals were housed and cared for under standard conditions and were euthanized in a CO_2 chamber after which the inguinal fat pad was harvested and ADSCs were isolated using previously published methods [139]. All subsequent steps were conducted under sterile conditions. Fat pads were rinsed twice in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Australia). The fat pad was then minced with a pair of scissors briefly till a fine slurry was formed, before digestion with collagenase type 1 (Gibco, Australia) for 45 minutes at 37°C. The suspension was then centrifuged at 1600g for 10 minutes at 4^{o}C to separate adipocytes from the stromal vascular fraction (SVF). The SVF pellet was resuspended in 3ml of DMEM and then pipetted carefully on top of 3ml of Ficoll-Paque PLUS (Sigma-Aldrich). Centrifugation at 1600g for 20 minutes removes red blood cells from the SVF. The ADSCs were then removed from the interface between the Ficoll-Paque PLUS and DMEM. This was then resuspended in 8ml of DMEM to dilute out residual Ficoll-Paque PLUS. The cells were washed twice in DMEM and centrifuged at 1000g. Upon completion of the final wash the pellet is resuspended in basal growth media DMEM Glutmax/F12 (Gibco, Australia) with 10% Fetal bovine Serum (FBS, Invitrogen). The suspension was then aliquoted into 2ml T-25 culture flask and incubated at 37^{o}C at 5% CO_2 for 2 days until ADSCs adhered to the culture flask. Non-adherent cells are eliminated by aspirating floating cells and replacing the media. ADSCs were passaged 3-5 times before utilised in experiments (Santos, 2014, PhD). Additionally, primary rat osteoblasts were donated by the Santos lab and cultured in basal growth media DMEM Glutmax/F12 (Gibco, Australia) with 10% Fetal bovine Serum until grown to confluency.

3.2.2 Assessment of rADSC viability during trypsin surface digestion *in vitro*

rADSC cell viability during trypsin surface digestion was assessed using PrestoBlue® Cell Viability Reagent (Thermo Fisher Scientific) in accordance to the manufacturer's protocol. In brief, rADSCs were coated onto a Nunc™ Nunclon™ 96 Well plate (Thermo Scientific, USA) at a cell density of 1×10^4 cells and treated with 50µg/ml of porcine trypsin (Thermo Scientific, USA) at predetermined time points of 5, 10, 15, 20 minutes. After each respective time point, the cells were washed with PBS and incubated with PrestoBlue® reagent and the relative cell viability assessed using absorbance spectroscopy at 570nm. The data obtained was subsequently loaded onto Microsoft Excel (Microsoft, USA) and cell viability was expressed as a percentage relative to the control group of untreated rADSCs. Statistical analysis was performed in Microsoft Excel between the variables with an ANOVA analysis.

3.2.3 Trypsin Digestion of rADSCs Surface

rADSCs were incubated with porcine trypsin for 15 minutes at 37ºC. Cells were pelleted and the resulting supernatant collected and further digested overnight with MS grade trypsin. Proteomic analysis of all biological samples underwent LC/MS/MS through an AB Sciex Q-Star Elite quadrupole time-of-flight mass spectrometer[204]. Subsequent peaks were searched using Mascot to obtain relevant protein identification

3.2.4 Biotinylation of rADSC surface

Intact rADSC cell surface proteins were grown to 80% confluency in a T-175 flask before being washed in PBS and then biotinylated with sulfo-NHS-LC biotin (Thermo Scientific) for 30 seconds(s) on ice. The samples were then quenched with 50 mM Tris-HCl, pH 7.4 for 15 mins before being washed twice in PBS. The cells were lysed and mechanically removed from the flask using a cell scraper, into solubilisation buffer (7 M urea, 2 M Thiourea, 40 mM Tris (pH 8.8), 1% (w/v) C7bZ0), followed by probe sonication at 50% power for 30s on ice. Proteins were reduced and alkylated with 20 mM acrylamide monomers, 5 mM TBP for 90 mins. Insoluble material was pelleted by centrifugation. Biotinylated proteins were purified by avidin column affinity

chromatography by incubating the lysate with avidin agarose (Thermo Scientific), collecting the flow through and washing 5 times in 100 mM NH_4HCO_3. The enrichment of biotinylated proteins was achieved via elution in 30% acetonitrile and 0.4% trifluoroacetic acid. Proteins were concentrated through a 3, 000 MW cut-off filter, followed by acetone precipitation and centrifugation to collect the precipitated protein. Biotinylated proteins were resuspended in SDS-PAGE sample buffer, separated by 1D SDS PAGE, transferred onto PVDF membrane and probed with ExtrAvidin; visualising proteins using diaminobenzidine. Duplicate samples were separated by 1D SDS PAGE, in-gel trypsin digested and analysed by LC/MS/MS through an AB Sciex Q-Star Elite quadrupole time-of-flight mass spectrometer and subsequent peaks were searched using mascot to obtain relevant protein identification.

3.2.5 Western blot of biotinylated surface proteins

Western blotting was utilised to verify the presence of biotinylated proteins after surface labelling with impermeable sulfo-NHS-LC biotin. Surface biotin labelled rADSC cell lysates were prepared and separated by 1-D SDS-PAGE using Bio Rad TGX gels and transferral to PVDF membrane, as described previously [286]. In brief, transferred proteins on PVDF membranes were blocked with 5% (w/v) skim milk powder in PBS with 0.1% Tween 20 (v/v) at room temperature for 1 hour, followed by incubation with ExtrAvidin(diluted 1:5000 in PBS-0.1% Tween) for 1 hour at room temperature. Blots were washed and developed using SIGMAFAST 3,3'-diaminobenzidine tablets (Sigma-Aldrich) as per manufacturer's instructions and then subsequently imaged with a photographic scanner (Epson, USA).

3.2.6 Cell coating of the hydroxyapatite

A cylindrical piece of converted hydroxyapatite (dimensions: 5mm length x 3mm diameter) was coated individually by separately placing each scaffold into the 15 mL Falcon tube containing 1mL of the cell suspension (1 $x10^6$ cells). The tubes were placed in a laboratory centrifuge (Eppendorf Centrifuge 5702) for 1, 2, 3, 4 and 5 minutes at 700 g to ensure efficient seeding density as previously published [202]. Following this the cap of the tubes were released slightly to allow gas exchange and

the tubes placed back in a 37°C incubator with 5% CO2. The cells were incubated at 37°C in 5% CO_2 atmospheric pressure and media replaced every 84 hours for a total of 14 days and 30 days when cells were harvested for analysis.

3.2.7 Ultracentrifugation-isolation (UC) of rADSCs membrane and subsequent solubilisation

Rat primary bone, basal rADSC and seeded coralline HA were harvested with TrypLE Express (Life Technologies, Australia) after 30 days. The collected cells were pelleted at 1000 x g after centrifugation for 10 minutes. The supernatant then removed and the pellet resuspended in 1% SDS, Triton X-100 solution and 5mM $MgCl_2$ to efficiently lyse cells and obtain all intracellular soluble proteins.

The resuspended solution was centrifuged at 16000rcf for 20 minutes at room temperature for the removal of insoluble membrane components.The membrane pellet obtained after lysing and centrifugation was washed in PBS, resuspended in a 1% SDS, Triton-X solution and 5mM $MgCl_2$, containing complete protease inhibitor tablets (Life Technologies, Australia) and probe sonicated on ice for 30 seconds. The resulting solution was further fractionated through ultracentrifugation at 162000 x g for 2 hours at 4°C. The obtained pellet was washed twice by being resuspended in PBS to remove any cytosolic contaminant proteins and ultracentrifuged again at 162,000g for 2 hours at 4°C.

The resulting pellet was finally resuspended in UTC7 (7M Urea, 2M Thiourea, 1% C7Bz0) and probe sonicated again for 30 seconds on ice three times until a homogenous mixture was obtained. The enriched samples were subsequently analysed by LC-MS/MS using an AB Sciex Q-Star Elite quadrupole-TOF instrument [204]. (Sciex, CA).

3.2.8 Membrane Proteomic Characterisation of Seeded and Non-Seeded Adipose Derived Stem Cell

All proteins obtained from the membrane enrichment protocols were reduced and alkylated with 5 mM tributylphosphine and 20 mM acrylamide for 90 minutes at room temperature before finally being quenched with 1mM dithiothreitol. A total of 100µg of protein was separated by SDS-PAGE and stained initially with Flamingo Fluorescent Gel Stain (Bio-Rad, Australia) followed by visualisation with colloidal Coomassie Blue G-250. The resolved proteins from the gel lanes were sliced into fourteen equal-sized pieces separated by 5mm and then destained using 50% Acetonitrile and 50% 100mM Ammonium Bicarbonate. Proteins were identified by in-gel trypsin digestion (overnight) followed by LC-MS/MS using an AB Sciex Q-Star Elite quadrupole-TOF instrument [204].

3.2.9 Proteomics MS/MS data analysis

Data obtained from MS/MS spectra were submitted to database search by using the MASCOT search engine (hosted by the Walter and Eliza Hall Institute for Medical Research Systems Biology Mascot Server) against the LudwigNR database (composed of the UniProt, plasmoDB, and Ensembl databases (vQ312. 19 375 804 sequences; 6 797 271 065 residues)) with the following parameter settings: fixed modifications: none; variable modifications: propionamide, oxidized methionine; enzyme: semitrypsin; number of allowed missed cleavages: 3; peptide mass tolerance: 100 ppm; MS/MS mass tolerance: 0.2 Da; charge state: 2+ and 3+, and species restriction of *Rattus norvegicus.*

The resulting MASCOT dat files were transferred to Scaffold software (v4.00.02, Proteome Software, Portland, OR) for further validation and comparison MS/MS-based peptide and protein identifications. Peptide identifications were accepted if their calculated probability assigned by the Protein Prophet algorithm was >95.0% with at least 2 unique peptides identified. Protein identifications were accepted if their calculated probability was >70.0%. Proteins with only 1unique peptide identification

were manually inspected for quality of spectra. The data obtained from this proteomic analysis was further characterised through TMpred to predict possible transmembrane domains within the protein [287].

Venn diagrams were constructed utilising the 'Quantify' tool on Scaffold and a comparison was made between the basal rADSCs, Rat Primary Bone and Seeded rADSC. Additional GO annotations were performed on identified proteins to obtain molecular function. Proteins of interest were assessed via a literature survey which involved the retrieval of full articles for their suitability for review. Language other than English, and manuscripts that did not include in their content the role of osteogenesis were used as an exclusion criteria.

3.3 Results

3.3.1 Subproteome analyses

Currently there are multiple techniques that be utilised to investigate the subproteome. Prior to committing to a particular subproteome analysis protocol, we assessed numerous methodologies such as trypsin shaving of surface accessible proteins, surface biotinylation of intact rADSC cells, and UC-isolation of rADSC membrane proteins in order to ascertain which methodology would produce a larger array of proteins.

3.3.2 Trypsin digestion of rADSC surface

Trypsin is a potent serine protease that if incubated with cells for a prolonged period, will cause cell lysis via complete proteolysis of the cell. A cell viability assay was performed to quantitatively measure the cytotoxicity of trypsin during digestion of the rADSC surface. Cytotoxicity can lead to the presence of cytosolic contaminants within our analysis. The predetermined time points of 5, 10, 15 and 20 minutes were chosen as previous studies have performed trypsin surface digestion for as long as 30 minutes [288]. No study has investigated the optimal surface trypsin digestion for ADSCs for mesenchymal stem cells.

The comparison of cell viability between PBS treated and Trypsin treated rADSCs showed there was no significant difference between the two forms of treatment during any of the time points (Figure 3.1). However, there was a significant difference between the 5 minutes and 20 minutes of surface trypsin digestion. Therefore, we selected 5 minutes' as the optimum contact time between trypsin and all surface accessible rADSC proteins while additionally minimising the possibility of cytosolic contaminants.

3.3.3 Western blot of biotin labelled surface proteins

To assess the extent of surface biotin labelled proteins, a Western blot of surface biotinylated proteins against ExtrAvidin was performed. The blot revealed the presence of biotinylated proteins within our elution lanes labelled 'E1-E4' (Figure3.2). The negative control of non-labelled rADSC lysate ('-') did not develop a reaction while

the positive control of biotinylated whole cell rADSC lysate (+) reacted intensely. However, the labelled protein banding pattern of (W1, W2, W3 and W4) is similar to the protein band profile of elutions indicating cytosolic contaminants may be present within our elutions, and that our recovery of biotinylated proteins was insufficient.

3.3.4 Proteomic comparison of subproteome techniques.

The rADSC subproteome was isolated from the three different techniques of trypsin surface digestion, biotinylation and UC-isolation. The data obtained was subsequently loaded into Scaffold, and Venn diagrams were constructed to compare the protein profile of each dataset (Figure 3.3). The LC/MS/MS analysis revealed a proteomic dataset of 52, 111 and 182 proteins identified for trypsin shaving, biotinylation and UC-isolation techniques respectively.

The proteomic comparison shows that a larger array of proteins were identified by the UC-isolation technique. However, both biotinylation and surface trypsin digestion identified 12 and 13 proteins respectively that UC-isolation did not. These proteins could possibly have more surface accessible tryptic sites thus increasing in abundance during the trypsin surface digestion procedure. This would lead to the presence of numerous proteins that were not within the dynamic range for the UC-isolation procedure. Due to the higher protein identification in the UC-isolation, we employed this technique for all our proceeding subproteome investigations of seeded HA membrane proteins.

Assessment of rADSC viability during *in vitro* surface trypsin digestion

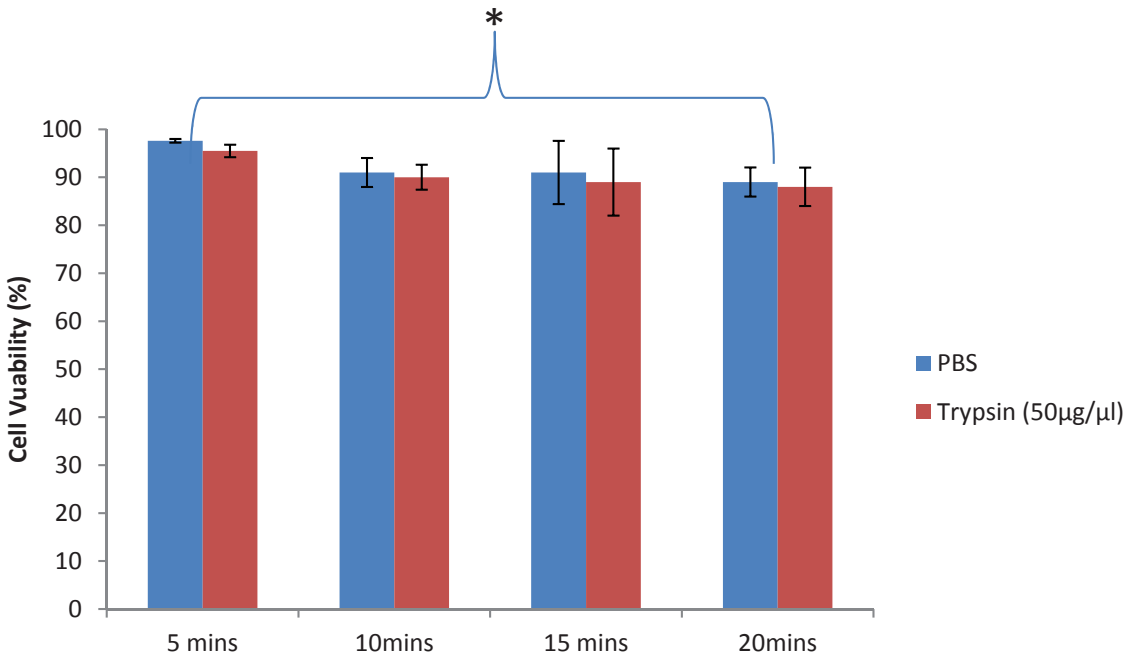

Figure 3.1: The associated cell viability between PBS and Trypsin treated rADSCs for 5mins, 10 mins, 15 mins and 20 mins. **Statistically significant ($p < 0.012$).*

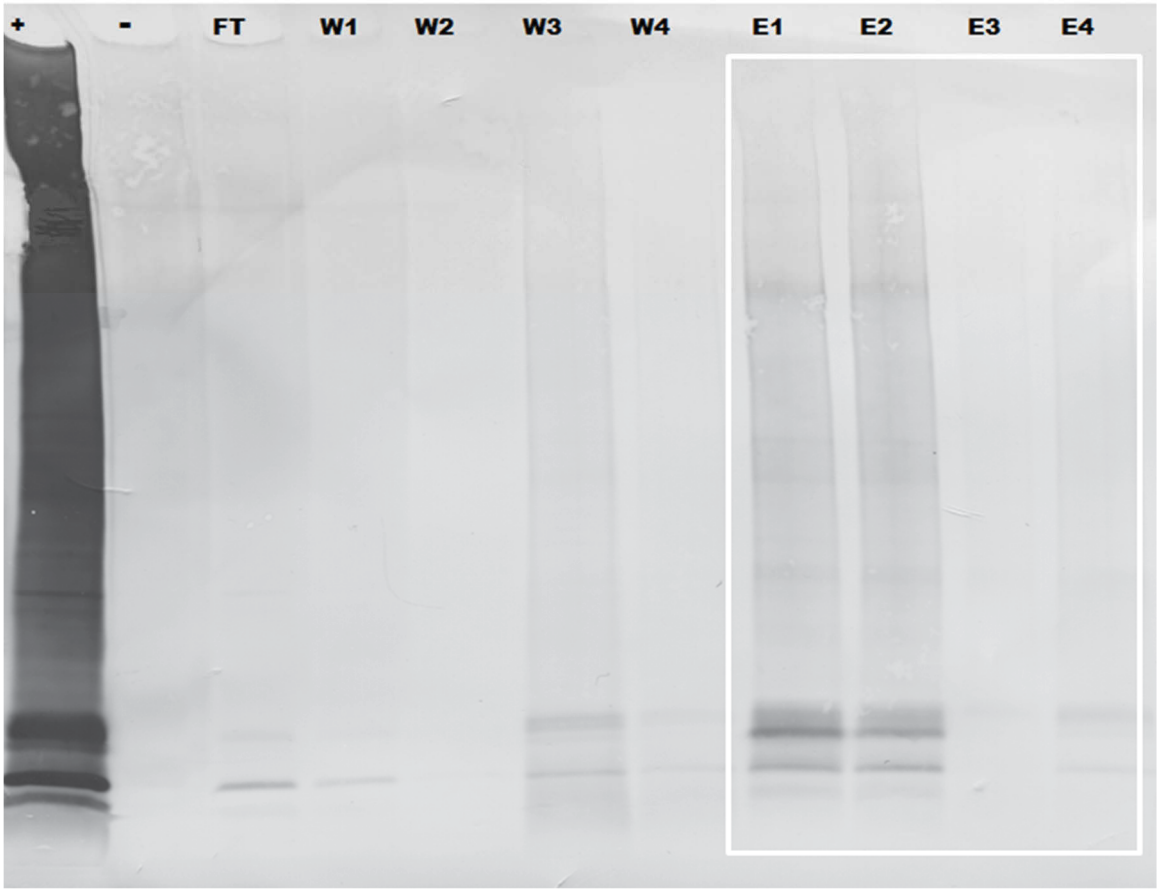

Figure 3.2: Western blot of biotin labelled rADSCs surface proteins against strepavidin-HRP. Biotinylated whole cell lysate is denoted by '+' . Proteins that naturally bound to avidin were tested for in lane '-'. A flow through of the avidin column is shown by lane 'FT'. The extensive washes were collected and blotted in lanes W1, W2, W3 and W4. Subsequent elutions of surface biotinylated proteins are shown in lanes E1. E2. E3 and E4 (white box).

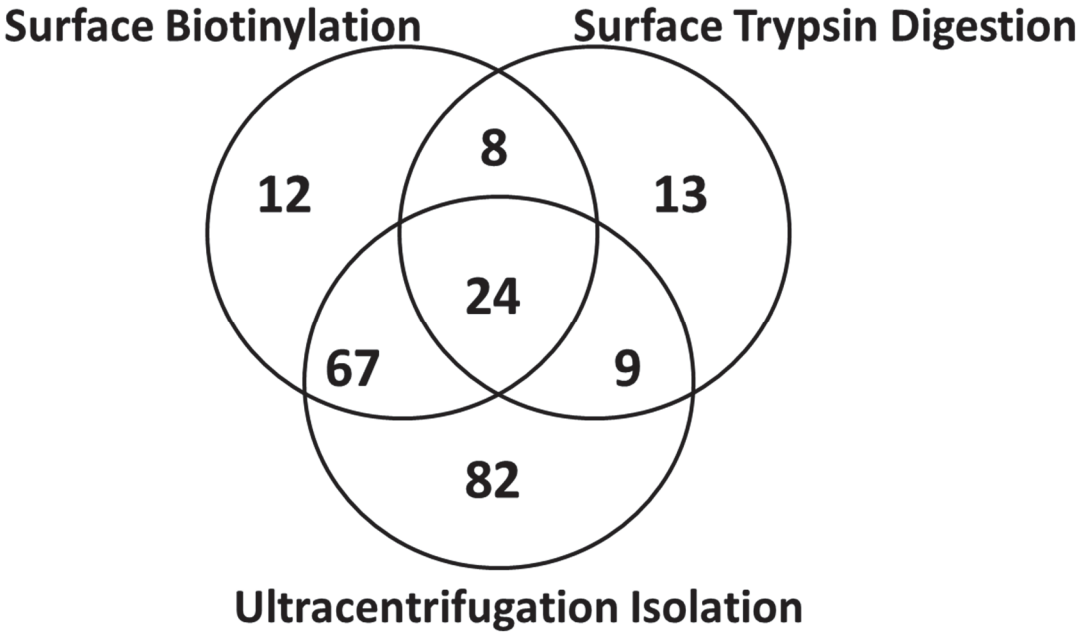

Figure 3.3: The proteomic comparison of different membrane isolation techniques. A total of 12 unique proteins were identified by surface biotinylation, 13 for surface trypsin digestion and 82 for UC-isolation (ultracentrifugation isolation).

3.3.5 Membrane Proteomic Comparison of Seeded and Non-Seeded ADSCs

In our previous study, we identified a number of osteogenic proteins from the soluble cytosolic component of seeded HA (Chapter 2), however the insoluble membrane fraction that remained was not analysed. Within this study, we aimed to investigate the insoluble membrane component of basal rADSCs, seeded HA rADSCs and rat primary bone cells. Furthermore, we aimed to compare the membrane proteomic profile of the three different cohorts and assess their osteogenic potential.

After the isolation of proteins with our preferential technique (Chapter 3.2.7), a total of 215 proteins were identified from membrane solubilisation of Seeded HA, 181 from basal rADSCs and 123 proteins from the rat primary osteoblast cohort. To classify any potential osteogenic proteins, we compared the protein profile of seeded HA with rat primary osteoblast with the inclusion of basal rADSCs and presented each respective association as a Venn diagram (Figure 3.4). Overall, 81 proteins were shared between all three cohorts, however only 12 proteins were found to be common between the seeded HA and rat primary osteoblast membrane and their possible role in osteogenesis was explored (Table 3.1). In brief, only 3 out of the 12 proteins shared between the two cohorts were found to be associated with bone formation and they included CD44, integrin beta-1, and protein Rab-1A.

In terms of unique membrane protein expression, up to 49 were expressed by seeded HA. The set of unique seeded HA membrane proteins were analysed for their possible role in bone morphogenesis through a literature survey. In total, out of the 49 proteins identified, 30 were considered to play a role in osteogenesis (Table 3.2). In summary proteins involved in tissue remodelling (basigin, pro-neuregulin and aggrecan core protein) and osteoclastic ECM proteins (flotillin-1, lactadherin and destrin) were identified. More importantly, a number of osteoblast ECM proteins such as fibronectin, collagen-6 alpha 1, veriscan core protein, synaptagmin-5, nidogen and periostin were also identified in the membrane of seeded HA rADSCs. Additionally, bone morphogenetic protein (BMP) regulatory proteins calnexin and calreticulin were also

present in the seeded HA membrane. Finally, our data revealed proteins associated with osteogenesis within the day 30 rADSC seeded cell population listed in table 3.2. In our analysis of these osteogenic proteins we detected 8 defined proteins involved in calcium ion regulation, an essential process of maintaining osteogenicity (Table 3.2). The presence of Annexin A4 indicated a response to bone formation as annexins are abundant in bone matrix vesicles, and are speculated to play a role in Ca^{2+} entry into vesicles during bone morphogenesis [208].

In summary, there were only a few membrane proteins shared between seeded HA and primary rat osteoblasts while a larger number of proteins were only identified in seeded CHA which included an array of osteoclastic, osteoblastic and extracellular matrix (ECM) proteins. Furthermore, the membrane proteome profile of rADSCs changed post seeding onto a HA biomaterial.

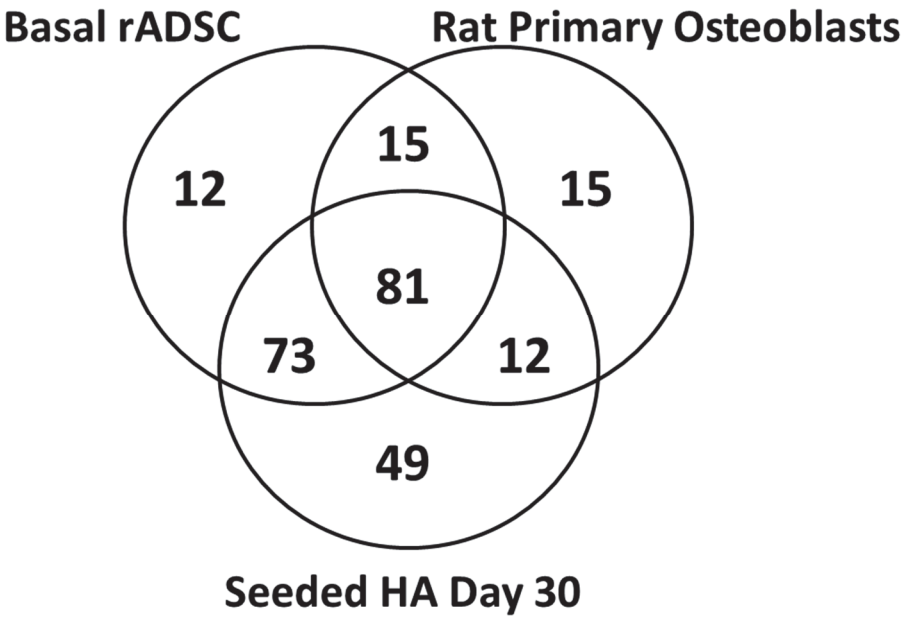

Figure 3.4: Venn diagram of rADSC membrane proteins identified between seeded HA Day 30, basal rADSCs and rat primary osteoblasts. As previously reported, all three cohorts shared a total of 81 membrane proteins while 49 proteins were uniquely identified in seeded HA.

Table 3.1: Listed are some of the proteins identified to be shared between Seeded HA rADSC and primary rat osteoblast membrane proteins with their respective number of annotated transmembrane domains and associated description. Proteins without an annotated TM domain is highlighted with a "*" and has their role within the membrane explored through literature surveys.

Accession	Protein Name	Transmembrane domain (Y/N) #	Protein Score	Coverage	Role in Bone Morphogenesis
P06761	78 kDa glucose-regulated protein	Yes (2)	180	38%	This protein facilitates the assembly and folding of proteins and multimeric complexes within the endoplasmic reticulum. Plays no significant role in bone morphogenesis.
F1M5X1	Protein Rrbp1	Yes (1)	61	4%	Located within the membrane of the endoplasmic reticulum, Rrbp1 mediated mRNA binding by acting as a ribosome receptor. Plays no role in osteogenesis.
P26051-2	Isoform of CD44	Yes (3)	30	6%	CD44 is a multifunctional adhesion molecule that binds to hyaluronic acid. type 1 collagen, and fibronectin. It is found to be expressed on the plasma membrane of differentiating osteocytes as it is a core component of ECM regulation during bone morphogenesis [289].
P61751	ADP-ribosylation factor 4	None*	138	48%	ADP-ribosylation factor is a small GTP-binding protein that is required for binding of the coatomer protein beta-COP to Golgi membranes. Plays no significant role in bone morphogenesis.

E9PU16	Ras-related protein Rab-1A	Yes (1)	155	26%	R-Ras promotes cellular adhesion through the regulation of the actin cytoskeleton [290]. Additionally, R-Ras has shown to interact with integrin beta-1 to mediate its cellular adhesion of osteoclasts [291].
P49134	Integrin beta-1	Yes (3)	47	4%	A dimer integrin protein that is expressed by osteoclasts to mediate adhesion [292].

Table 3.2*:* Listed are some of the proteins identified in the Seeded ADSC membrane proteome involved in osteogenic regulation with their respective number of annotated transmembrane domains and associated description. Proteins without an annotated TM domain is highlighted with a "*" and has their role within the membrane explored through literature surveys.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
Q6P2A7	Flotillin 1	Yes (1)	3% (1)	Flotillin-1 is a membrane protein that has been distributed exclusively in a lipid raft membrane fractions in various cells. During osteoclastogneogenesis, the raft component flotillin are increased after stimulation with macrophage colony stimulating factor [293].
F1LST1	Fibronectin	Yes (3)	4% (3)	Fibronectin has been shown to regulate human mesenchymal stem cell osteogenesis through their interaction with integrin chains on the surface. An antibody which inhibits fibronectin has further elucidated a negative feedback mechanism on osteoblast activity [294,295].
D3ZUL3	Collagen VI alpha 1	Yes (1)	2% (2)	Collagen VI is an essential ECM protein in cancellous bone as it's deficiency has been reported to reduce bone mass. The reduced bone mass was caused by a distortion in the morphology of osteoblasts and their subsequent dysfunction in collagen re-arrangement. Therefore, collagen VI is important to

				maintain bone production [296]
Q9ERB4	Versican core protein	Yes (2)	1% (2)	Versican core protein is heavily expressed in woven bone during development and moderately expressed in lamellar bone of 6 week old rats. Additionally, it is an extracellular matrix protein which binds hyaluronic acid and aids in cellular differentiation and motility [297].
Q68FP1-2	Isoform 2 of Gelsolin	Yes (1)	11% (5)	Isoforms of gelsolin are an actin regulating protein for osteoclast precursor cells [241]. During osteoclast maturation, osteopontin regulates the interaction of gelsolin to form cytoskeletal rings important for osteoclast formation during bone remodeling [298].
P70490	Lactadherin	Yes (2)	3% (1)	Also known as MFG-8, is a negative regulator of osteoclast function. It is commonly upregulated during osteoclast differentiation to control the rate of resorption and prevent pathological bone loss [299].
P55260	Annexin A4	N/A	5% (1)	During skeletal development of annexins is well-characterized in bone as components of mineralizing matrix vesicles[300]. Annexin A4 is a calcium dependent phospholipid membrane binding protein modulates NF-κβ signalling by directly interacting with the p50 heterodimeric p50/65 NF-κβ complex which participates in the RANKL pathway of osteoclast

				regulation [301] [302].
P26453-2	Isoform 2 of Basigin	Yes (2)	4% (1)	Basigin (CD147), also known as extracellular matrix metalloproteinase inducer is annotated to be a surface expressed glycoprotein that activates matrix metalloproteinase and vascular endothelial growth to stimulate expression of molecules involved in tissue remodeling and angiogenesis [303]
P15800	Laminin subunit beta-2	Yes (2)	10% (13)	Lamnin has shown to be a cofactor in promoting osteodifferentiation of rat bone marrow stem cells (rBMSCs) and bone formation within porous HA *in vivo* [304]. Additionally, laminin is a well characterized glycoprotein that aids in proliferation and adhesion of osteoblast like cells [305].
P08494	Matrix Gla protein	Yes (1)	24% (4)	Matrix Gla protein (MGP) is a BMP regulatory protein that binds and inhibits and activity of BMP2, 4 and 7 to reduce excess calcification during bone remodeling [306]. MGP is part of the vitamin-K dependent, Gla-containing family proteins and is present in bone tissue as it plays a role in bone organisation [307].
P07897-2	Isoform 2 of Aggrecan core protein	Yes (3)	1% (1)	Also known as cartilage-specific proteoglycan core protein, it is an integral ECM protein found in cartilaginous tissue that allowed the rigours of joint loading on bone [308]. *In vivo,*

				Aggrecan is upregulated which consequently increases osteogenesis in a rat femur model [309].
P04937-2	Isoform 2 of Fibronectin	Yes (3)	25% (21)	Essential for osteoblast mineralisation, fibronectin and all its associated isoforms are involved in the compaction osteoblast compaction through cell mediated fibrillogenesis in the ECM of skeletal bone. Additionally, fibronectin participates in the modulation of type I collagen secretion by osteoblasts during osteogenesis.
O70244	Cubilin	Yes (11)	1% (1)	Cubilin binds 25-hydroxycholecalciferol [25(OH),D3] for vitamin D homeostasis in vivo. Mutations In cubilin and subsequent loss of function humans cause vitamin D deficiency and gradual impairment of bone morphogenesis.

O35569-2	Isoform 2 of Pro-neuregulin-2, membrane-bound isoform	Yes (2)	1% (1)	Functionally cleaved into neuregulin-2 which is a member of the epithelial growth factor (EGF) family of proteins responsible for fibroblast differentiation and is a key regulator of angiogenesis during tissue damage [310].
Q66HF2	Synaptotagmin-5	Yes (9)	3% (1)	Synaptotagmin-5 mediates calcium-dependent binding at synaptic vesicles for calcium-induced exocytosis of essential ECM molecules in differentiating osteoblasts [311].
P27615	Lysosome membrane protein 2 (LAMP-2) [CD107b]	Yes (2)	12% (4)	Similar to lysosome membrane protein-1 (LAMP-1), LAMP-2 translocates from the lysosomal membrane compartment to the surface membrane when platelets are activated. Regulated surface expression of these heavily glycosylated proteins may play a role in the adhesion and prothrombotic phenotype of these cells during the formation of a hematoma.
D3ZAF5	Periostin, osteoblast specific factor (Predicted), isoform CRAa	Yes (1)	16% (8)	The Rattus norvegicus isoform of the protein Periostin-like factor, it is commonly expressed in mesenchymal stem cells of the periosteum and proliferating osteoblasts. Additionally, Zhu et al found that periostin is secreted and produced by osteoblasts *in vitro* to accelerate osteoblast proliferation and differentiation for bone morphogenesis *in vivo* [312].

F1LM84	Nidogen-1	Yes (5)	15% (13)	Also known as enactin, Nidogen-1 is a basement membrane protein that interacts with fibronectin, collagen 1, vitronectin and collagen IV *in vitro* during osteogenesis, Additionally, Eslaminejad *et al* showed enactin can influence proliferation and differentiation of osteoblasts *in vitro* [313].
P97532	Mercaptopyruvate sulfurtransferase (MST)	Yes (2)	7% (2)	MST acts as a sulfur carrier, for downstream metabolic purposes. This process is essential for the down regulation of NAD(P)H+ oxidase, in reducing the oxidative stress on osteoblastic cells during high metabolic activity [314].
O70513	Galectin-3-binding protein (G3BP)	Yes (4)	3% (1)	G3BP contains binding sites for transcriptional factors essential for osteogenesis such as galectin 3 [315], type V and VI collagens, β integrins, and fibronectin [316]. Due to the substrate specificity of G3BP, it has been proposed to act as a promoter for bone formation. For example, Stock *et al* has shown that galectin-3 expression is controlled by Runx2 during osteoblast differentiation and skeletal development [315].
P11762	Galectin-1	None	18% (2)	Galectin 1 is expressed in the cytoplasm and nucleus of proliferating and differentiating osteoblast cells [317]. It's highly associated within the nuclear matrix during osteoblast differentiation[317]. The presence of galectin-1 on the transmembrane of precursor mesenchymal stem cells has previously shown to play a role in the formation of chicken limb during skeletogenesis [318].

F1MAN8	Laminin, alpha 5, isoform	Yes (5)	17% (45)	The expression of laminins on the surface of human mesenchymal stem cells is limited by the spatial dimensions of their growth environment [319]. Importantly, *in vivo* and *in vitro* studies have outlined laminin, alpha 5 to recruit and adhere osteoblasts during osteodifferentiation[320] .
Q9QZK5	Serine protease HTRA1	Yes (2)	26% (11)	Human serine protease HTRA1 positively regulates osteogenesis of human bone marrow-derived mesenchymal stem cells and mineralization of differentiating bone-forming cells through the modulation of extracellular matrix protein [321].
Q7M0E3	Destrin	N/A	24% (3)	Destrin regulates disassembly and assembly of actin filaments which are essential for cellular membrane ruffling and extension, a process common in osteoblast differentiation into osteocytes during bone development[322].
P51799	H+/Cl-exchange transporter 7 (CIC7)	Yes (10)	1% (1)	During bone resorption, CIC-7 prevents intracellular polarization through proton secretion and extrusion of chloride ions within the ruffled border membranes of osteoclasts. Additionally, the subsequent HCL formed in the resorptive space dissolves the hydroxyapatite portion of the bone matrix [323].
P35565	Calnexin	Yes (9)	2% (1)	Calnexin is a calcium binding protein commonly expressed within the microvesicles of osteoblast like cells, called Saos-2 cells. It's thought that calnexin is an important component in the mineralisation process of HA

				within the lumen of differentiating Saos-2 cells [324].
P18418	Calreticulin	Yes (1)	17% (6)	Calreticulin is a calcium binding protein that binds to HA, and forms a complex with calnexin and calmodulin to sequester influxes of calcium during the bone mineralisation process [324].
Q32KJ9	Arylsulfatase G	Yes (2)	2% (1)	Arylsulfatase is a novel family of sulfatases that is essential for degradation and remodeling of sulfate esters within the ECM.
Q66HD0	Endoplasmin	Yes (2)	7% (3)	During calcium deposition, endoplasmin, along with other calcium binding chaperone proteins translocate towards the extracellular matrix.. This mechanism continues to be important in maintaining intracellular and extracellular calcium homeostasis[324] .

3.4 Discussion

Previous research has investigated the MSC membrane to obtain a biological perspective for its appropriate usage. An earlier work by Granéli *et al*. in 2013 used a stable isotope labelling by amino acids in cell culture (SILAC)-based approach to quantitatively investigate differentially expressed surface markers in osteo-differentiated human bone marrow stromal cells (hBMSCs) [303]. The group identified a number of membrane associated proteins including 11 differentially expressed CD markers, two of which, CD92 and CD10 were found to be highly expressed post chemically induced osteogenic differentiation. Similar proteomic approaches have also been used to track the changes in the expression patterns of human mesenchymal stromal cells (hMSCs) membrane proteins during osteoblast differentiation, such as Niehage *et al* who introduced a biotinylation approach in enriching plasma membrane proteins. The group identified over 200 plasma proteins in undifferentiated hMSCs and 41 CD markers, several of which were not previously identified in both osteo-differentiated and basal ADSCs [325].

Our first aim was to establish the effectiveness of trypsin digestion of surface accessible proteins, biotinylation of intact rADSC and rADSC membrane UC-isolation and solubilisation. The inherent issue with membrane investigations is the possibility of cytosolic contaminants. We verified rADSC cell viability during surface trypsin digestion procedure using Prestoblue and demonstrated that 15 minutes of surface digestion does not lead to cell death. To date, no study has verified the optimal conditions for surface trypsin digestion of adipose derived stem cells. Trypsin digestion is commonly employed in prokaryotic organisms due to the feasibility of surface exposed tryptic sites on prokaryotic organisms in comparison to larger and more multimeric surface exposed proteins present on eukaryotic organisms [326].

However, this inherent issue does not occur for surface labelling with sulfo-NHS-LC biotin due to its small spacer arm length (13.5 angstroms) allowing greater accessibility to surface exposed proteins than trypsin. The major disadvantage however is the

specificity of sulfo-NHS-LC biotin as it can only target lysine and arginine residues similarly to trypsin. Furthermore, nonspecific binding between proteins and the avidin column was evident in our Western blot of labelled proteins where the banding profile of our wash and elutions were overserved to be similar.

Ultracentrifugation isolation of membrane proteins yielded a wider array of rADSC membrane proteins than biotin labelling and trypsin digestion. Therefore, we pursued the UC-isolation and solubilisation strategy to investigate the dynamic changes undergone by the rADSC membrane proteome before and after seeding onto HA. Our study revealed that seeding rADSCs onto HA for 30 days allowed the expression of over 30 osteogenic membrane proteins, indicating that HA might promote osteogenic activity upon seeded rADSCs as revealed by our previous study (Chapter 2).
Interestingly, the majority of the unique seeded HA membrane proteins are involved in the formation of the bone extracellular matrix such as laminin subunit beta-2,matrix Gla protein, periostin, Nidogen-1, Galectin-3-binding protein (G3BP and Serine protease HTRA1. A study found serine protease HTRA1 to be a positive regulator of MSC osteogenesis where its presence is essential for the efficient mineralisation of differentiating bone cells [327].

Similar observation could be made for proteoglycans and secreted proteins identified in our HA membrane analysis such as periostin, osteoblast specific factor and nidogen-1**.** Periostin, osteoblast specific factor is the *Rattus norvegicus* isoform of periostin like-factor which is located in mesenchymal cells of the periosteum and osteoblasts lining trabecular bone [328]. Additionally, PLF has shown to be expressed and secreted by osteoblasts *in vitro* as a matricelullar protein which promotes osteoblast proliferation and differentiation [312]. Nidogen-1 is a sulfated glycoprotein which is ubiquitously expressed in the mesenchyme during embryonic limb development [329]. It's role within the ECM was the subject of Kruegel and co-workers study which demonstrated that nidogen-1 binds to fibronectin through the RGD domain to influence skeletal limb development [330].

However, not all identified unique seeded HA membrane ECM proteins were involved in osteogenesis, rather some were essential cell adhesion molecules involved in cellular attachment to surfaces such as laminin subunit beta 2. For example, Yoshikawa *et al* demonstrated that laminin coated onto porous HA greatly contributed to the attachment and osteo-differentiation of bone marrow stem cells, in comparison to fibronectin coated, seeded porous HA scaffolds [331]. Additionally, laminin isoforms influence progenitor cell migration *in vitro* within the bone marrow study [332]. This could possibly explain the reason laminin subunit beta 2 was only identified in our seeded HA membrane cohort and not in primary rat osteoblasts as the adhesion to the porous HA architecture was mediated by the expression of laminin.

Some of the calcium binding proteins such as calnexin and calreticulin were also in our seeded HA membrane analysis. Calnexin is an integral membrane chaperone proteins that is commonly expressed within the microvesicles of osteoblast Saos-2 cells where it mediates the mineralisation process of HA within the lumen of differentiating Saos-2 cells [333]. Calreticulin is an endoplasmic reticulum protein that has shown to play a role in osteogenesis [334] however its presence within our membrane analysis does highlight a disadvantage with our isolation procedure. As Melo-Braga and co-workers have previously shown, UC-isolation cell fractionation to purify microsomal membranes, which include not only plasma membranes but also many other intracellular membrane bounded compartments [335] . For instance, Foster et al. who purified membranes on density gradients found only <75 bona fide cell surface proteins among the 463 proteins identified [27] required for maintaining stem cells in a given niche. However, this could be seen as a latent advantage as it allows us to analyse a diverse array of proteins not previously permissible with our previous technique which disregards any insoluble material during proteomic analysis.

3.5 Conclusion

In conclusion, we assessed trypsin digestion of rADSC surface proteins, biotin-labelling of intact rADSCs and UC-isolation of the rADSC 'insoluble' fraction in an attempt to analyse the rADSC subproteome. We determined UC-isolation to be the suitable method for subproteome analysis as it obtained the highest number of protein identifications with the widest array of proteins that were previously not detected by our initial shotgun proteomics approach.

The subproteome of seeded rADSCs revealed the presence of matrix deposition proteins that have shown to facilitate osteogenesis, receptor proteins that can potentially bind to bone morphogenic growth factors and a number of integrins and laminins that may possibly aid in cellular attachment to the 3-Dimensional matrix of the coralline scaffold. Additionally, the detection of a number of proteins that are constituents of intracellular membrane bounded compartments gave us a novel perspective of the proteins expressed within organelles during HA seeding.

Therefore, the proteomic analysis of the seeded rADSC subproteome improved our understanding of membrane associated proteins that play a role in the attachment and proliferation of ADSCs onto coralline HA matrices. Within our study we also identified a number of ECM proteins that play a role in osteogenic biology supporting our previous study that HA may influence ADSCs towards an osteogenic lineage. These findings may in turn help advance ADSCs towards a sufficient therapeutic role in the bone tissue engineering.

Chapter Four: (Paper III): The *in vitro* characterisation of Multiple Coralline Scaffolds Seeded with Human Adipose Derived Stem Cells for Tissue Engineering.

Declaration

I declare that the following publication included in this thesis in lieu of a chapter meets the following:

- More than 50% of the content in the following publication included in this chapter has been planned, executed and prepared for publication by me
- The work presented here has been prepared for publication and will be submitted

- The initial draft of the work has been written by me and any subsequent changes in response to co-authors and editors reviews was performed by me
- The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis.

Publication title: *The in vitro characterisation of Multiple Coralline Scaffolds Seeded with Human Adipose Derived Stem Cells for Tissue Engineering*
Authors: Krishneel Singh, Jerran Santos, Benjamin B. A. Raymond, Besim Ben-Nissan, Matthew P Padula , Bruce Milthorpe.
Candidate's contribution (%): above 50 %
Journal name: Journal of Tissue Engineering and Regenerative Medicine Volume/ page numbers:
Status: To be Submitted:

I declare that the publication above meets the requirements to be included in the thesis.

Candidate's name: Candidate's signature: Date:

4.1 Introduction

Natural scaffolds derived from biological tissues exhibit ideal structures that offer optimal bioactive potential such as host cell attachment, integration and proliferation [336]. A favourable orthopaedic scaffold is one that best mimics the inorganic properties of natural bone and contains interconnected macro and micro pores that allow essential chemokines and cytokines into the fracture site for efficient bone regeneration. Bioceramics have attempted to emulate the aforementioned properties of bone repair, however only a few have adequately copied the structural components of bone [337-339].

Over the past few decades, coralline bioceramics have emerged as favourable candidate for bone tissue engineering due to its porous structure which ranges from 150μm to 300μm and its ability to form chemical bonds with bones and soft tissues *in vivo*. Consisting of aragonite (a calcium carbonite), coral mineral is known to be biodegradable and has been shown to contain micropores throughout the flat regions of its architecture [340]. However, coralline mineral has weak mechanical properties as it is brittle and cannot withstand loadbearing weights during locomotion of target fracture sites [188].

Various methods have been utilised for the augmentation of coralline material, including a hydrothermal conversion process that converts aragonite into hydroxyapatite (calcium phosphate). This conversion process produces coralline hydroxyapatites (cHA) with increased similarity to bone material while maintaining its previously mentioned ideal morphology for bone reconstruction [187,201]. Furthermore, the hydrothermal process has been performed on a number of Australian corals from the genus Goniopora and Porites. The cHA obtained from the genus Goniopora is produced with a pore size of 250μm and interconnectivity of 200μm whilst still maintaining its distinct properties of high strength in tangential and longitudinal direction [188,201].

Similarly, cHA sourced from the genus Porites has been hydrothermally converted to produce a bioceramic with a pore size of 150µm and pore interconnectivity size of 144µm with distinct favourable mechanical properties that are similar to that of cancellous bone [341]. However, a different species of Porites that was concurrently hydrothermally converted by Hu and co-workers [201] produced a bioceramic with less pore interconnectivity and a pore size of 130µm. This unique property of less pore interconnectivity and a smaller pore size gives the coralline bioceramic a higher structural density, which is a trait that has previously never been investigated in the field of bone tissue engineering. Density of bone plays an important role in daily loading stress which shares a relationship with the cyclic strain force [342]. Compact bone has high density to withstand the cyclic strain force experienced during everyday activity [343] and thus a bioceramic with less pore interconnectivity is considered advantageous as it provides an alternative to coralline bioceramics that mimic cancellous bone.

Additionally, the hydrothermal process can include a second phase of nano coating which facilitates the nanopores with hydroxyapatite to increase the mechanical strength of the coralline scaffold, as shown by Ben-Nissan and co-workers[187]. This second phase nano coating process overcomes the weak biaxial strength of unconverted calcium carbonate and first phase converted HA while maintaining its osteoconductive and bioactive properties[187].

The seeding of converted HA was thoroughly investigated in Chapters 2 and 3 of this thesis which determined converted HA to be bioactive as it allowed cellular attachment, integration and proliferation of rat adipose derived stem cells (rADSCs). Furthermore, the data indicated that rADSCs begin to take on an osteoblast like phenotype due to the presence of a number of osteogenic proteins. Additionally, the number of osteogenic proteins identified increased after further incubation of 30 days, a finding that has not been previously reported.

Within this study, the aim was to shift the cellular model of seeding from rADSCs to human sourced ADSCs (hADSCs) to create a more translational approach of utilising HA for human therapeutic usage. Currently, hADSCs have been utilised for tissue engineering as they have the capacity to differentiate into tissues of the mesenchyme such as cartilage, muscle and bone. Previous hADSCs applications would initially involve direct administration of stem cells into the target site [344], however current models prefer guiding bone regeneration through three dimensional scaffolds. Recently, a natural coral scaffold was successfully seeded with allogenic hADSCs to repair a canine cranial model [283]. Additionally, hADSCs seeded onto a collagen coated hydroxyapatite scaffold and induced with osteogenic media promoted osteogenesis *in vitro* [345]. Similarly, coralline hydroxyapatite coated with various growth factors was a key focus of another study which found converted hydroxyapatite coated with insulin growth factor 1 promoted *in vivo* bone regeneration in a rabbit bone defect [2].

In spite of this work, the mechanism through which cHA induces bone regeneration is not understood. Furthermore, coralline cHA can be fabricated through multiple methods, with each variation producing a significantly new biomaterial and there is little knowledge on the role these materials could play in the field of bone regeneration engineering.

Therefore, in the present investigation we have made an attempt to evaluate the effectiveness of unconverted coralline calcium carbonate and multiple hydrothermally converted corals (Table 4.1) as a carrier of hADSCs to promote osteoconductivity and osteoinduction *in vitro*. Our hypothesis is that the natural porous architecture of coral scaffolds enables hADSC attachment, proliferation and ultimately osteodifferentiation. The effect of coralline material on hADSCs was assessed with a proteomic based approach through tandem LC/MS/MS and cell viability after 30 days of seeding was investigated using SEM. Furthermore, the cell culture medium was analysed for cytokine, chemokine and growth factors with a Bio-plex Pro™ system.

The results obtained from this study confirmed that hADSCs seeded onto coralline scaffolds were able to proliferate *in vitro* and proteomically obtain an osteocyte like phenotype without the influence of external growth factors and chemokines. Additionally, the different pore sizes of converted coralline hydroxyapatite influenced a phenotypic change within the hADSCs with a number of osteoclast like proteins being expressed. Furthermore, second phase nano coating with HA plays an important role in allowing a larger repertoire of osteogenic proteins to be expressed after 30 days *in vitro*.

4.2 Methods

4.2.1 Fabrication and characterization of converted porous coral scaffold

Sea coral from the genus Goniopora and Porites was converted to coralline hydroxyapatite utilising hydrothermal exchange process (Table 4.1) [187,201]. The converted corals were sliced into irregular shapes and washed thoroughly using distilled water (MilliQ™) to remove soluble salts. After 30 minutes of U.V sterilisation,the corals were weighed and prepared for seeding with cells.

Table 4.1: *The type of coralline material utilised within this study with their respective genus source, pore size and stage of hydrothermal conversation before hADSC seeding. Their mechanical properties have been further evaluated in another study* [346].

Type of Coralline material	Genus	Pore Size	Phase of hydrothermal conversation
Unconverted coralline carbonate	Porites	150 µm	N/A
Converted cHA	Porites	150 µm	First phase
Macroporous cHA	Goniopora	250 µm	First phase
High Density cHA	Porites	130 µm	First phase
Nanoporous cHA	Porites	144 µm	Second phase nano-coating with hydroxyapatite

4.2.2 Isolation and culture of plastic adherent hADSCs.

Subcutaneous abdominal adipose tissue was donated from a healthy female patient under human ethics approval from the human ethics research committee (UTS HREC 2015000118). All subsequent steps were conducted under sterile conditions. Adipose-derived stem cells (ADSCs) previously isolated from patient lipoaspirates by the Santos lab (UTS HREC 2015000118-SANTOS) were revived from cryogenic storage in Dulbecco Modified Eagle Medium (DMEM)/F-12 (Gibco) + 10% v/v foetal bovine serum (Gibco) in

T25 tissue culture flasks. Cells were grown at 37°C, 5% CO_2 and expanded into T75 and subsequently T175 tissue culture flasks when proliferated to at least 80% confluency. Cells were then continuously expanded into additional T175 tissue culture flasks until Passage 9 (P9). At P9, cells were either harvested or utilized for neuronal differentiation. Media was replaced every 3.5 days during growth and maintenance of ADSCs.

4.2.3 Culture of hADSCs on Hydroxyapatite

Irregular pieces of converted and unconverted coralline calcium carbonate were coated individually by separately placing each scaffold into a 15 mL Falcon tube containing 1mL of cell suspension (1 x10^6 cells). The tubes were placed in a laboratory centrifuge (Eppendorf Centrifuge 5702) for 1, 2, 3, 4 and 5 minutes at 700 g which has previously shown to ensure efficient seeding density on cHA [347]. The seeded coralline scaffolds were placed in a 37°C incubator with 5% CO2 atmospheric pressure and the culture medium replaced every 84 hours for a total of 30 days before being subsequently harvested for analysis.

4.2.4 Proteomic Characterisation of hADSCs seeded onto coralline scaffolds

Cells adhered to seeded scaffolds (converted, unconverted coralline calcium carbonate, high density cHA, macroporous and nanoporous cHA) were harvested with TrypLE Express (Life Technologies, Australia) after 30 days. The collected cells were pelleted at 1000 x g after centrifugation for 10 minutes. The supernatant then removed and the pellet resuspended in 1% SDS, 0.5% Triton X-100 solution and 5mM $MgCl_2$ to efficiently lyse cells and obtain all intracellular soluble proteins. The resuspended solution was centrifuged at 16000rcf for 20 minutes at room temperature for the removal of insoluble membrane components (chapter 4.2.5).

The proteins in the resulting supernatant were assayed for total protein content. The obtained proteins were reduced and alkylated with 5 mM tributylphosphine and 20 mM acrylamide for 90 minutes at room temperature before being quenched with 1mM dithiothreitol. A total of 100µg of protein was separated by SDS-PAGE and

stained initially with Flamingo Fluorescent Gel Stain (Bio-Rad, Australia) followed by visualisation with colloidal Coomassie Blue G-250. The gel lanes were sliced into four equal-sized pieces and then destained using 50% Acetonitrile in 100mM Ammonium Bicarbonate. Proteins were subjected to in-gel trypsin digestion (overnight) and then pooled into one complex mixture before proteomic analysis via LC-MS/MS using a Sciex 6600 TripleTOF hybrid quadrupole-time-of-flight mass spectrometer (section 4.2.6).

4.2.5 Membrane enrichment and solubilisation of seeded hADSCs

The membrane pellet obtained after lysing and centrifugation (section 4.2.4) was washed in PBS, resuspended in a 1% SDS, 0.5 % Triton-X solution and 5mM $MgCl_2$, containing complete protease inhibitor tablets and probe sonicated under ice for 30 seconds. The resulting solution was further fractionated through ultracentrifugation at 162000 x g for 2 hours at 4°C. The obtained pellet was washed twice by being resuspended in PBS containing protease inhibitor tablets to remove any cytosolic contaminant proteins and ultracentrifuged again at 162,000g for 2 hours at 4°C. The resulting pellet was finally resuspended in UTC7 (7M Urea, 2M Thiourea, 1% C7Bz0) and probe sonicated again for 30 seconds under ice three times until a homogenous mixture was obtained. The enriched samples were pooled with their respective seeded hADSC cytosolic fraction (chapter 4.2.4) and subsequently analysed by the LC-MS/MS using an AB Sciex TripleTOF hybrid quadrupole-time-of-flight mass spectrometer (Sciex, CA).

4.2.6 LC/MS/MS.

Using an Eksigent 415 autosampler connected to a 415 nanoLC system (Eksigent, USA), 5 μL of the sample was loaded at 300nl/min with MS buffer A (2% Acetonitrile + 0.2% Formic Acid) by direct injection onto a laser pulled nanoflow column (75 μmID x 150 mm;) packed with C18AQ resin (1.9μm, 200A, Dr Maisch, Germany). Peptides were eluted from the column and ionised into the source of a 6600 TripleTOF hybrid quadrupole-time-of-flight mass spectrometer (Sciex, CA) using the following program: 2-35% MS buffer B (80% Acetonitrile + 0.2% Formic Acid) over 90 minutes, 35-95% MS

buffer B over 9 minutes, 95% MS buffer B for 9 minutes, 80-2% for 2 min. The eluting peptides were ionised at 2300V. An Intelligent Data Acquisition (IDA) experiment was performed, with a mass range of 350-1500 Da continuously scanned for peptides of charge state 2+-5+ with an intensity of more than 400 counts/s. Up to 50 candidate peptide ions per cycle were selected and fragmented and the product ion fragment masses measured over a mass range of 100-2000 Da. The mass of the precursor peptide was then excluded for 15 seconds.

4.2.7 Proteomics MS/MS data analysis

The MS/MS data files produced by the 6600 TripleTOF were searched using Mascot Daemon (version 2.4; [348]) against the MSPnr100 database (based on the major reference sequence repositories of NCBI, Refseq, UniProtKB, EupathDB and Ensembl. Duplicate entries at the species-level are removed, “multispecies” entries (NCBI) and “fragments” (TrEMBL) are ignored. vQ115) with the following parameter settings. Fixed modifications: none. Variable modifications: propionamide, oxidised methionine, deamidated asparagine. Enzyme: semitrypsin. Number of allowed missed cleavages: 3. Peptide mass tolerance: 50 ppm. MS/MS mass tolerance: 0.1 Da. Charge state: 2+, 3+ and 4+. The results of the search were then filtered by including only protein hits with at least one unique peptide (Bold Red) and excluding peptide hits with a p-value greater than 0.05.

Peptides identified by Mascot were further validated using Scaffold (v4.4, Proteome Software, Portland, OR) and by manual inspection of the MS/MS spectra for the peptide to ensure the b- and y-ion series were sufficiently extensive for an accurate identification. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 80.0% probability assigned by the Protein Prophet algorithm [349]. Venn diagrams were constructed utilising the ‘Quantify’ tool on Scaffold and a comparison was made between the multiple cHA scaffolds. Additional GO annotations was performed on identified proteins to obtain molecular function and biological processes

of certain proteins of interest using the PANTHER blast tool [350].

Proteins of interest were assessed via a literature survey which involved the retrieval of full articles for their suitability for review. Language other than English, and manuscripts that did not include in their content the role of osteogenesis were used as an exclusion criteria

4.2.8 Scanning Electron Microscopy of Seeded Adipose Derived Stem Cells

After 30 days of incubation, culture medium was removed and the scaffolds were washed 3 times in PBS. Samples were fixed in 2% glutaraldehyde and 5% formaldehyde in PBS at 4°C overnight. Samples were further washed in PBS and then dehydrated in a graded ethanol series of 20%, 30%, 50%, 70%, 80%, 90%, 96% and two exchanges of 100% ethanol. A 1:1 mixture of absolute ethanol:hexamethyldisilazane (HMDS) was then added followed by two exchanges in 100% HMDS [Sigma-Aldrich], allowing the final volume to dry completely for at least 24 hours. Scaffolds were mounted onto specimen stubs with adhesive carbon tape and silver paint (ProSciTech, Australia) and coated in a ~10 nm layer of carbon using a MED 010 Balzers Union Carbon Coater. Samples were imaged on a Zeiss Supra 55VP using the in lens detector at voltages of 2 or 5 kV.

4.2.9 Cytokine and Chemokine analysis of Seeded Adipose Derived Stem Cells culture medium.

An aliquot of 100 µl of conditioned media from the seeded hADSC samples was collected every 72 hours after initial seeding (Chapter 4.2.2) for 720 hours. The conditioned media samples were centrifuged at 16000 x g to pellet debris. The supernatant was collected and stored at -80°C until they were assayed in technical duplicates. Concentrations of IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17A, Eotaxin, FGF basic, G-CSF, GM-CSF, IFN-g, IP-10, MCP-1(MCAF), MIP-1a, PDGF-bb, MIP-1b, RANTES, TNF-α and VEGF were simultaneously evaluated using commercially available multiplex magnetic bead-based sandwich immunoassay kits (Bio-plex™ MAGPIX, Bio-Rad Laboratories). Assays were performed following the manufacturer's instructions. Briefly, 27 distinct sets of fluorescently dyed

beads loaded with capture monoclonal antibodies specific for each cytokine to be tested, were used. Secretion samples (50 µl/well) or standards (50 µl/well) were incubated with 50 µl of pre-mixed bead sets into the wells of a 96 well microtiter plate (Bio-plex Pro flat bottom plates 96 well, Bio-Rad Laboratories) under shaking at 850rpm (IKA3, Sigma).

After incubation and washing, 25 µl of detection antibody mixture was added for 30 minutes at room temperature and the samples washed and incubated with 50ul of streptavidin-PE at room temperature under shaking for 10 minutes. After incubation and further washing, the samples were resuspended in 125µl assay buffer for 30 seconds before being assayed using the Bio-plex™ multiplex system (Bio-Rad Laboratories). Results were compiled and analysed in Bioplex manager version 2 and subsequently Microsoft Excel 2016 where all values were normalised against the secretion profile of basal hADSCs as a control.

4.3 Results

4.3.1 Proteomic characterisation of Seeded cHA and unconverted coralline calcium carbonate.

To identify the differences in protein expression of hADSCs after seeding on cHA and unconverted calcium carbonate, hADSCs were incubated for 30 days on multiple coralline scaffolds (Table 4.1) and proteins subsequently solubilised for LC/MS/MS analysis. Identification was based upon presence or absence from our control group of basal hADSCs. After the removal of proteins based upon our statistical exclusion criteria (Chapter 4.2.7), a total of 397 proteins was identified from our proteomic analysis of Seeded Converted Coralline and 321 proteins from our basal hADSC cohort. Furthermore, the LC/MS/MS analysis generated a set of 370, 118, 254 and 160 proteins for High Density, Macroporous, Nanoporous, Converted and coralline calcium carbonate.

The identified proteins were analysed for their possible role in bone morphogenesis through a literature survey. In total, over 250 detected proteins were considered to play a role in osteogenesis between all Seeded cHA cohorts. Of interest, 70 osteogenic proteins were uniquely expressed within the Seeded cHA dataset (Supplementary Table 4.1), 18 osteogenic proteins in our seeded unconverted coralline calcium carbonate (Supplementary Table 4.2), 41 osteogenic proteins in our macroporous cHA dataset (Supplementary Table 4.3), 44 proteins of osteogenic interest after seeding on nanoporous cHA (Supplementary Table 4.4) and 32 proteins were found to play an important role in bone morphogenesis post seeding onto high density cHA scaffolds (Supplementary Table 4.5).

Interestingly, up to 42 osteogenic proteins were identified to be shared between our Seeded converted cHA and unconverted coralline calcium carbonate (Supplementary Table 4.6) while 29 potential bone morphogenic proteins were present in our basal 30 day hADSC samples (Supplementary Table 4.7).

To compare the proteomic profiles of Seeded Unconverted coralline calcium carbonate

and Converted cHA, Venn diagrams were constructed to reveal 250 proteins to be uniquely present after cHA conversion in comparison to the 13 found to be only expressed in unconverted coralline calcium carbonate while over 140 proteins were shared between both samples (Figure 4.1). The literature review of the 250 proteins expressed from hADSCs after converted cHA treatment revealed 50 that play a role in bone morphogenesis with a short list provided (in Supplementary Table 4.8). The presence of Lamin A/C, clathrin heavy chain 1, stonin-2, actin related protein, chloride intracellular channel protein 4 and CamKII are found to play a role in calcium absorption as revealed by our literature review (Table 4.2). Additionally, the presence of adhesion molecules such as CD44, Talin-1, alpha actinin 4 and integrin alpha V constituted 36% of the annotated molecular function of the unique converted cHA proteins (Figure 4.2).

The literature review of the 13 unique proteins expressed by unconverted coralline calcium carbonate treated hADSCs (Supplementary Table 4.9) revealed the presence of a number of calcium binding protein synaptotagmin 1, osteo-regulatory proteins Collagen 1 alpha 2 chain, Moesin and insulin like growth factor 2 receptor (Table 4.3). GO annotation for the molecular function of the aforementioned proteins showed 30% to be involved in binding and 15% in structural integrity activity with only 5 gene IDs being matched with the 13 accession numbers provided (Figure 4.3).

The comparison of macroporous cHA and basal hADSCs indicated that only 10 unique proteins are expressed by hADSCs seeded onto macroporous cHA (Figure 4.4). The function of these unique seeded macroporous proteins was tabulated (Supplementary Table 4.10). To summarise, a majority of macroporous proteins are involved in actin production and folding through the expression of Actin-related protein 2/3 complex subunit 4, fascin homolog 1 actin-bundling protein. Destrin and T-complex protein 1 subunit zeta (Table 4.4). The other subset of proteins included some unique attachment proteins laminin subunit gamma-1 (Lγ-1) and a calcium sensor protein S100-A6 (Calcyclin) which allows cells to recognise and endocytose calcium. The GO annotation of the biological processes of these proteins showed 31.8% involved in

cellular processes and 18.2% in cellular component organisation, most due to the presence of actin regulatory proteins (Figure 4.5).

To compare the effect of nanoporous cHA on hADSCs, the proteomic profiles of converted cHA and basal hADSCs were used as controls in a Venn diagram to isolate proteins uniquely expressed by the nanoporous architecture of cHA (Figure 4.6). In total, 30, 23 and 61 unique proteins were identified to be expressed by nanoporous cHA, converted cHA and basal ADSCs respectively. The 30 unique proteins were further sorted by Protein class via GO annotation (Figure 4.7), their roles in bone morphogenesis explored through a literature survey and listed (Supplementary Table 4.11). A number of calcium binding proteins such as S100-A4, S100 A6, plectin, nucleobindin-1 and calnexin were identified (Table 4.5). The GO annotation further confirmed that up to 36.4% of proteins are involved in calcium binding. Cytoskeletal proteins possessed the second largest annotated classification for the proteins identified from nanoporous seeded cHA (24.1%) however the literature review shows cytoskeletal proteins Lamin B1 and integrin alpha V provide structural integrity of osteogenic tissue (Figure 4.8)

Finally, high density cHA was compared with basal hADSCs and not converted cHA as converted cHA is sourced from a different coralline genus. The properties of high density cHA induced the expression of 83 unique proteins in comparison to 34 proteins unique to the basal hADSC cohort (Figure 4.9).

Subsequent GO annotation analysis of the unique seeded high density cHA proteins revealed that 28.6% were annotated as cytoskeletal proteins and 14.3% are enzyme regulators and signalling molecules (Figure 4.10). A further literature review of the aforementioned proteins identified important osteo-regulatory molecules through the presence of NARS a crucial protein for osteoblast survival, DDX and Annexin 4, both of which are catalysts for the Runx3 induced osteoblast differentiation, and nucleobindin-1 which is involved in the exocytosis of calcium to form hard tissue (Table 4.6). Additionally, the literature survey of the proteins identified did reveal a number of

osteoclast proteins from our multiple coralline cHA cohorts.

Unlike seeded converted coralline scaffolds, the majority of the proteins in high density cHA were annotated as structural integrity proteins and extracellular binding proteins such as lamin B1, procollagen-2, FK506 binding protein 10 and Filamin B (Table 4.7). More notably, converted cHA expressed annexin A4, flotillin-1,filamin A, gelsolin, talin-1, biglycan, profilin 1, moesin and carbonic anhydrase, all of which play a role in either regulating or initiating osteoclastogenesis (Supplementary Table 4.1 and Table 4.8).

In summary, hydrothermally converted coralline scaffolds such as nanoporous cHA, macroporous cHA, high density cHA and coralline hydroxyapatite expressed a number of serine protease inhibitors, extracellular matrix (ECM) proteins, and proteins involved in osteogenic regulation. The number of proteins expressed between each of the cohorts differed and interestingly more osteogenic proteins were identified in hydrothermally converted coralline scaffolds than unconverted calcium carbonate. Overall, the proteome profile of hADSCs changed post seeding onto a cHA biomaterial and the proteomic signatures were significantly different between each cohort.

Evaluation of hADSC attachment and proliferation *in vitro*

4.3.2 Electron microscopy

SEM was employed to evaluate cell growth *in vitro* on our seeded unconverted coralline calcium carbonate and converted macroporous, nanoporous and high density coralline scaffolds. High density cHA samples revealed uniform pores of 130µm in diameter with a monolayer of cells observed adhering to and proliferating across the space between the interconnected pores (Figure 4.11). The surface topography of nanoporous cHA showed sufficient nano coating over the flat regions of the scaffold. Additionally, hADSCs were able to infiltrate the porous structure and partially fill the gap between each interconnected pore (Figure 4.12). Converted cHA showed similar seeding structures within its porous structures at different magnifications (Figure 4.13). Unconverted coralline calcium carbonate showed a less visible presence of efficient seeding with scarce populations of proliferating cells across the interconnected pores however, the morphology of the monolayer observed across the pore surface appeared granulated (Figure 4.14). Notably, we did not observe hADSCs proliferating across the large pores of macroporous cHA, however, at a higher magnification, cellular growth was observed across the surface of the macroporous scaffold (Figure 4.15).

Similarly to the SEM data obtained from Chapter 2.3.2, only a few cells aggregated on the granular surface of the coralline scaffold however the monolayer of cells observed across the pores shared a fibroblastic morphology [351] and their diameters were approximately 20µm wide. The presence of mono-layered cells over pores was consistent throughout the imaged SEM seeded HA.

4.3.3 Cytokine and Chemokine analysis of Seeded Adipose Derived Stem Cells culture medium.

At various time points corresponding to days 0, 3, 6, 9, 12, 15, 18, 21, 14, 27 and 30, 100 µl of conditioned medium was collected and stored before being subjected to BioPlex analysis as mentioned in 4.2.9. The secretion profile of all seeded coralline scaffolds were normalised against the secretion profile of hADSCs and their relative detectable concentration categorised as either 'Very low', 'Low', 'Medium' or 'High' in pg/ml.

The Bioplex analysis showed IL-5 was undetectable while IL-6 and IL-8 were found to be highly expressed across all seeded coralline secretion profiles (Supplementary Table 4.12-Supplementary Table 4.16). However, the concentration of IL-8 and IL-6 was the highest during the first 6 days post initial seeding, before decreasing over time (Supplementary Table 4.12-Supplementary Table 4.16). Additionally, low levels of Hu IL-1b, Hu IL-2, Hu IL-9, Hu IL-10, Hu IL-15, Hu Eotaxin, Hu IP-10, Hu MIP-1a, Hu PDGF-bb and Hu MIP-1b was detected across all seeded coralline scaffolds and moderate to high levels of Hu MCP-1(MCAF) was detected across all our data cohorts as well (Supplementary Table 4.12-Supplementary Table 4.16).

Interestingly, the secretion profile of converted cHA detected uniquely high levels of IL-17A (from 1003 pg/ml to 602 pg/ml) and Hu FGF basic (from 773 pg/ml to 568 pg/ml) (Supplementary Table 4.12 and Table 4.9). Alternatively, moderate levels of Hu G-CSF and Hu VEGF were similar in concentration in unconverted coralline calcium carbonate after 30 days.

The secretion profile of unconverted coralline carbonate showed unique moderate levels of IL-1ra, FGF basic and IFN-γ which also diminished over time, plateauing at 82pg/ml and 139 pg/ml for FGF basic and IL-1ra respectively (Supplementary Table 4.13 and Table 4.10). The highest reported cytokine for unconverted coralline calcium carbonate (IL-6 and IL-8) was similar between all five different coralline scaffolds.

A comparison between nanoporous cHA and macroporous cHA secreted profile

detected high levels of IL-1ra ,IL-6, IL-8 and Hu VEGF between the two cohorts (Table 4.11 and Table 4.12) . However, G-CSF was expressed in high levels only in nanoporous cHA while Hu MCP-1 was highly expressed in macroporous with a peak value of 2132 pg/ml and 4897 pg/ml respectively (Supplementary Table 4.14 and Supplementary Table 4.15). Furthermore, similar moderate levels of Hu IL-12, Hu IL-17A, Hu GM-CSF and Hu IFN-γ was detected between nanoporous and macroporous cHA with the exception of MCP-1 being only expressed at moderate levels in nanoporous cHA and FGF basic within the macroporous cHA cohort (Supplementary Table 4.13 and Table 4.10).

Finally the secretion profile of high density cHA revealed GM CSF to be uniquely expressed at high concentrations at 965 pg/ml on day 3 and 373 pg/ml on day 30 (Supplementary Table 4.16 and Table 4.12). Additionally, the chemokine G-CSF was also detected at a high concentration in high density cHA with a peak concentration 3766 pg/ml similarly to nanoporous cHA which had a peak G-CSF concentration of 2132 pg/ml (Supplementary Table 4.16). Interestingly, the chemokine RANTES was only detected at a moderate concentration on seeded high density cHA during day 3 at 627 pg/ml before dissipating till day 30 (Supplementary Table 4.16).

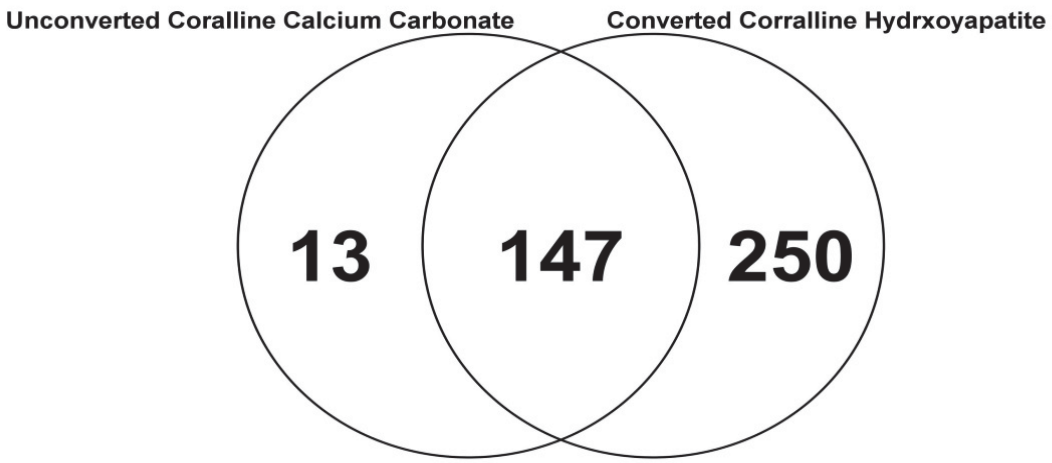

Figure 4.1: Venn diagram of proteins identified between Converted HA seeded ADSCs (Converted HA), unconverted coralline calcium carbonate seeded ADSCs and basal hADSCs. A total of 437 proteins were identified between all three cohorts, with 51 proteins uniquely expressed in Converted HA and 136 proteins shared between all three cohorts.

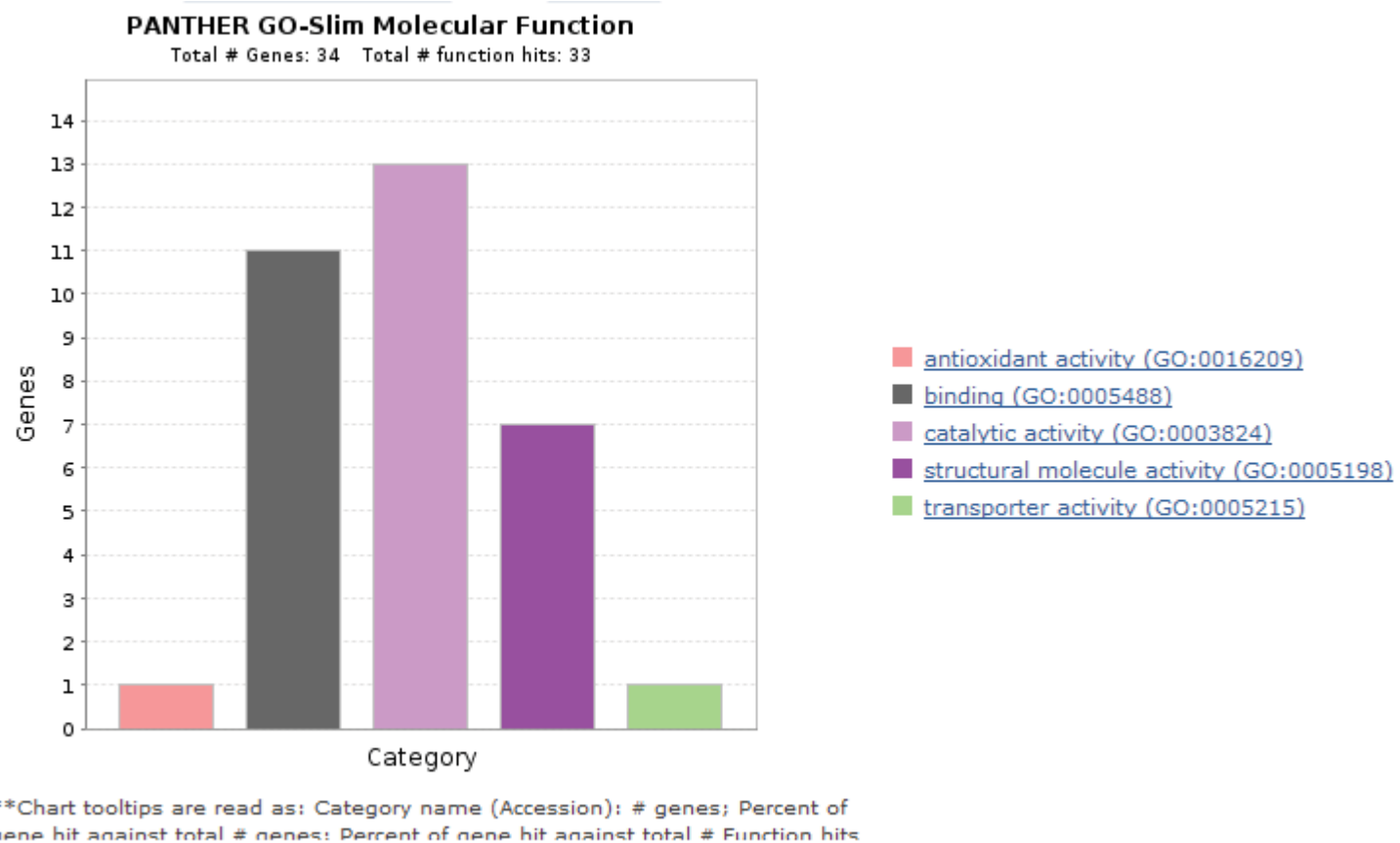

Figure 4.2: The annotated GO molecular function of proteins expressed after converted cHA treatment with the number of annotated genes on the y-axis and category of molecular function on the x-axis with the legend corresponding in colour. It can be seen that a large number of proteins are annotated to be involved in structural molecule activity and binding.

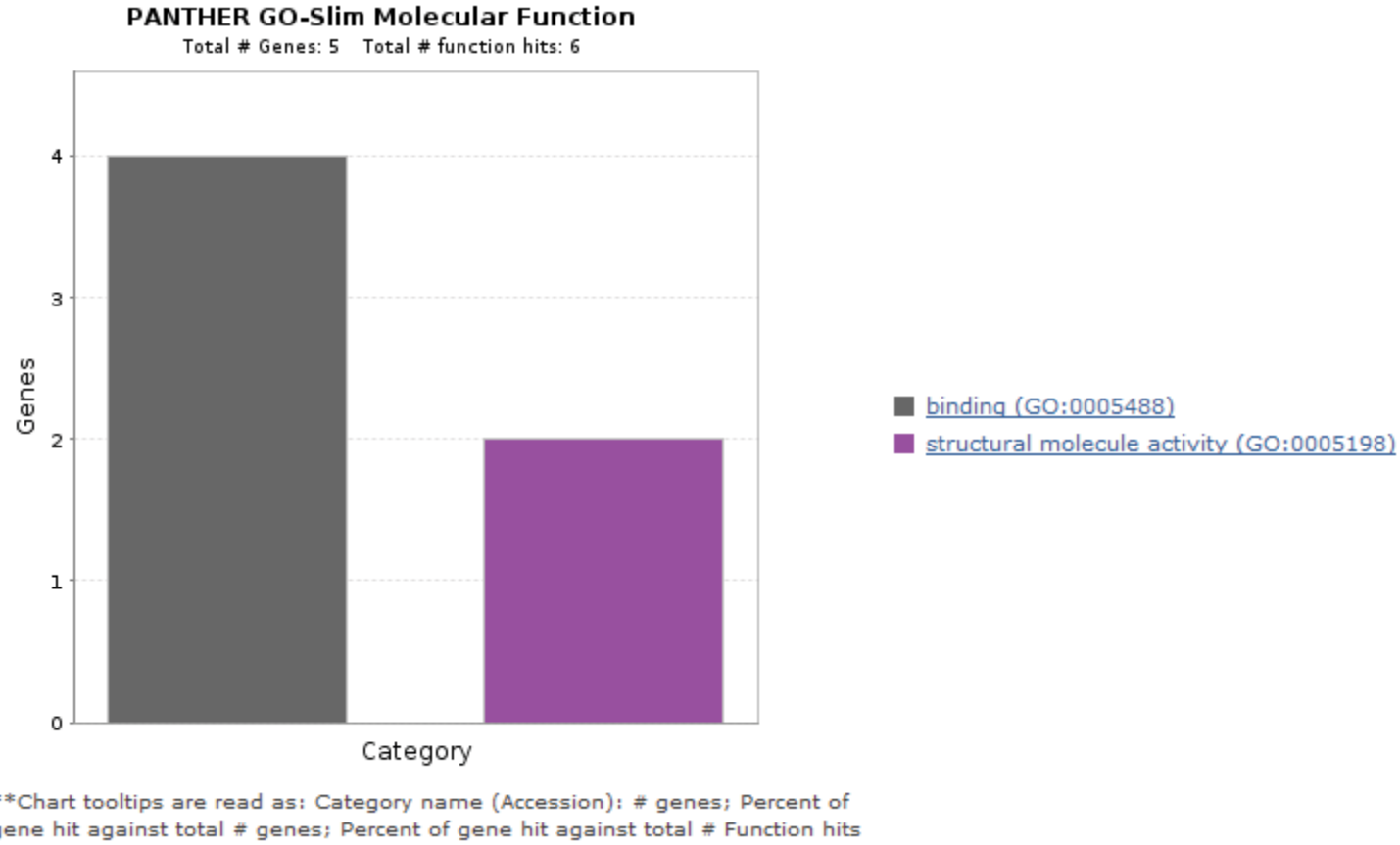

Figure 4.3: The annotated GO molecular function of proteins expressed after unconverted coralline calcium carbonate treatment with the number of annotated genes on the y-axis and categorising molecular function on the x-axis with the legend corresponding in colour. It can be seen that a large number of proteins are annotated to be involved in binding.

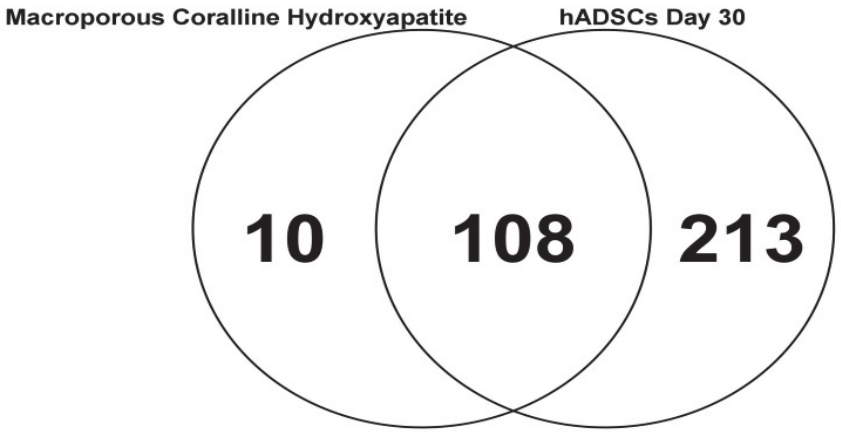

Figure 4.4: Venn diagram of proteins identified between Macroporous cHA seeded hADSCs (Macroporous Coralline Hydroxyapatite) and basal hADSCs (hADSCs Day 30). A total of 331 proteins were identified and a 108 proteins shared found the two data cohorts. Post seeding onto macroporous cHA led to the expression of 10 unique proteins.

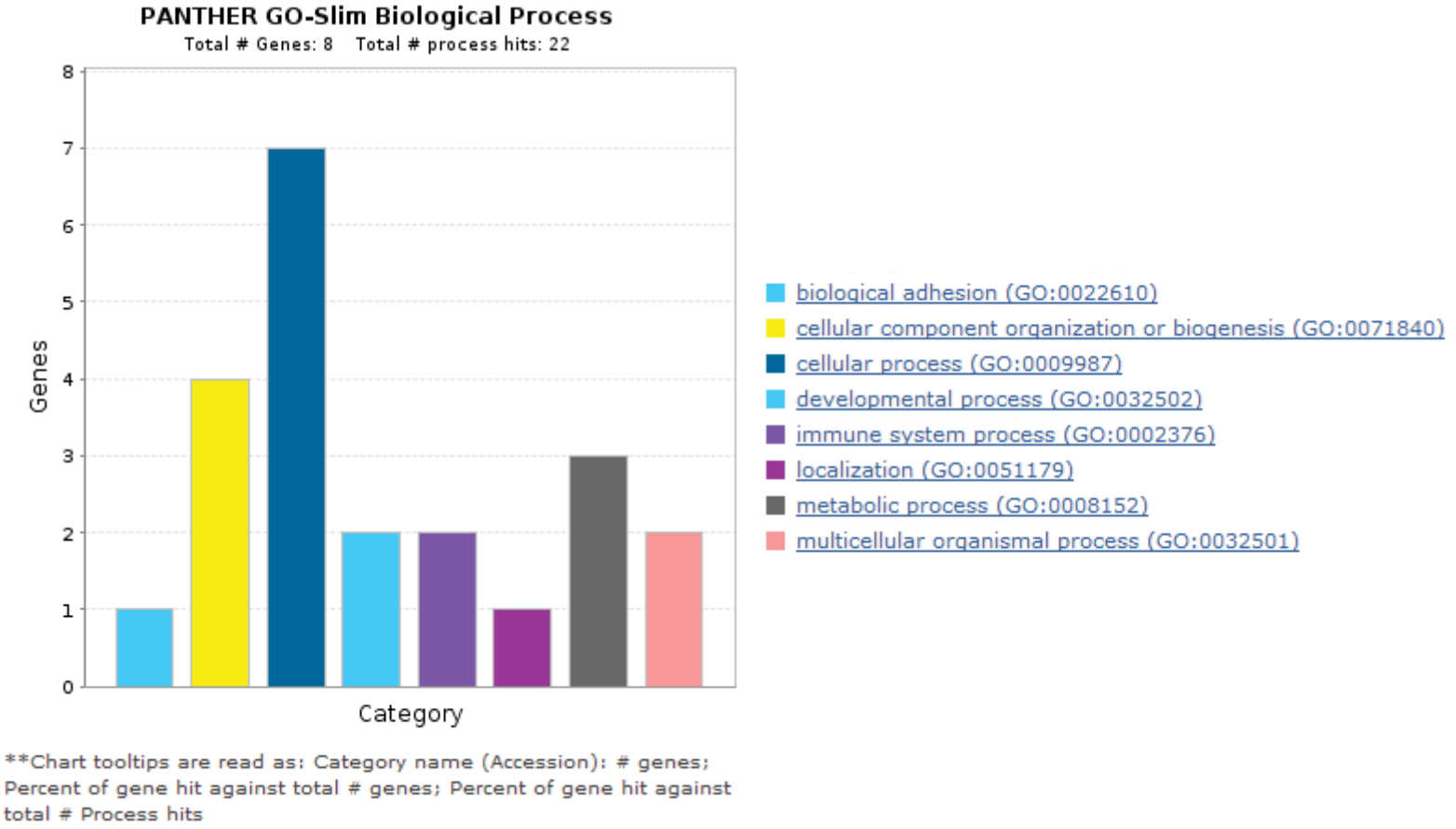

Figure 4.5: The annotated GO biological process of proteins expressed after macroporous cHA treatment with the number of annotated genes on the y-axis and categorising molecular function on the x-axis with the legend corresponding in colour. It can be seen that a large number of proteins are annotated to be involved in catalytic activity and binding.

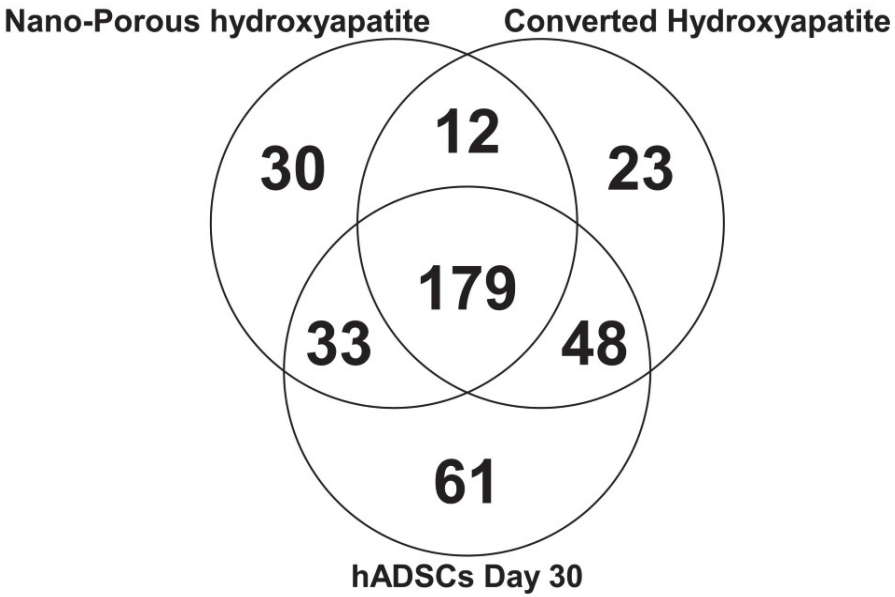

Figure 4.6: Venn diagram of proteins identified between Nano-porous cHA seeded hADSCs (Nano-Porous hydroxyapatite), Converted hydroxyapatite and basal hADSCs (hADSCs Day 30). A total of 386 proteins was identified with 30 unique proteins to be expressed after seeding onto nanoporous cHA and 179 proteins shared between all three cohorts.

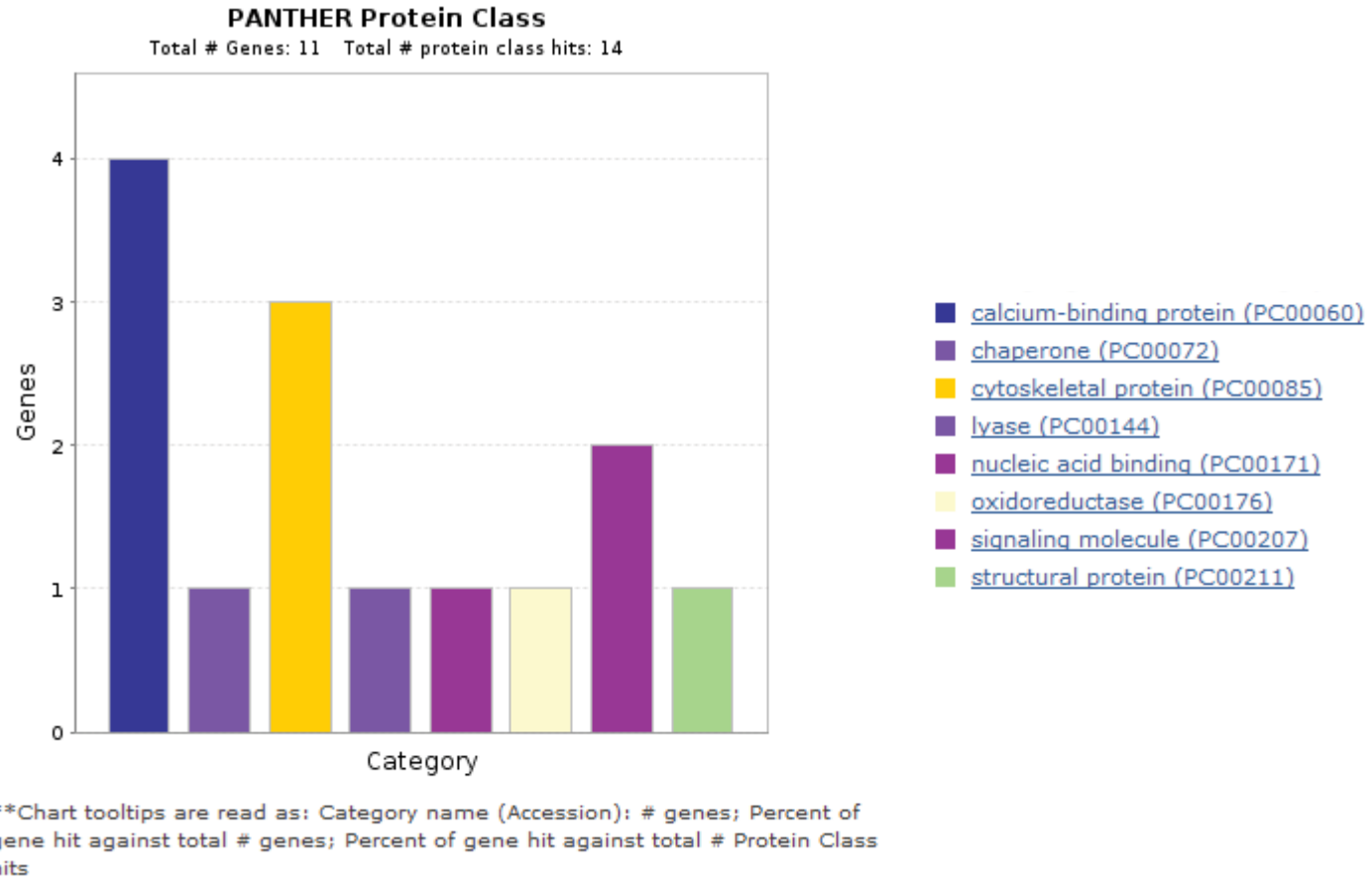

Figure 4.7: The annotated GO molecular function of proteins expressed after nanoporous cHA treatment with the number of annotated genes on the y-axis and categorising molecular function on the x-axis with the legend corresponding in colour. It can be seen that a large number of proteins are annotated to be involved binding.

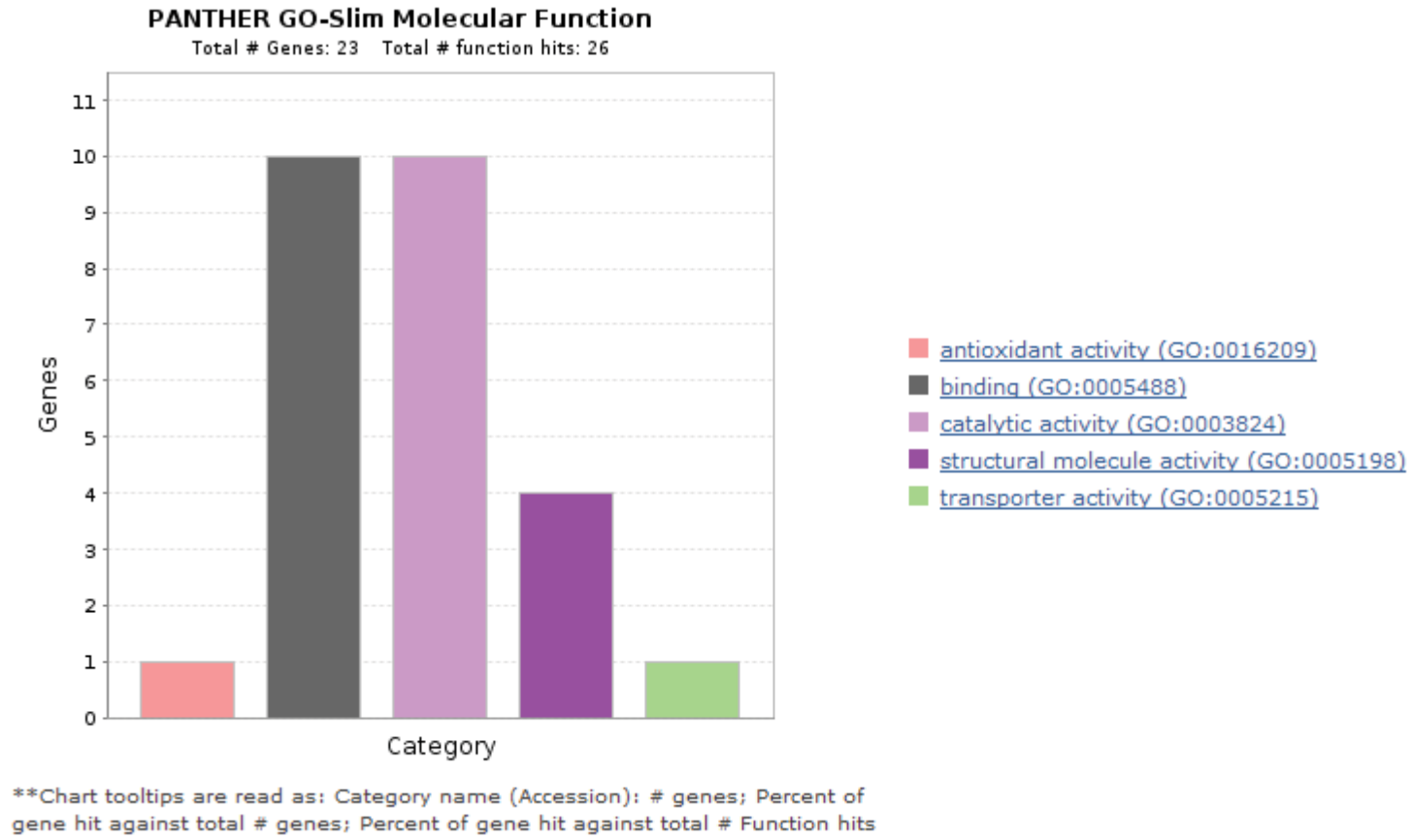

Figure 4.8: The annotated GO molecular function of proteins expressed after nanoporous cHA treatment with the number of annotated genes on the y-axis and categorising molecular function on the x-axis with the legend corresponding in colour. Interestingly, catalytic activity and binding is the most abundant molecular function annotated within this dataset.

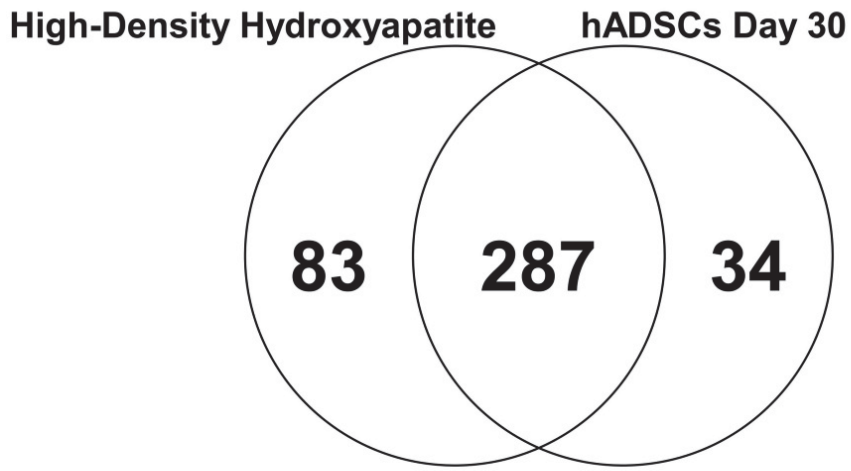

Figure 4.9: Venn diagram of proteins identified between High-Density HA seeded ADSCs (High Density hydroxyapatite) and basal hADSCs (hADSCs Day 30). A total of 413 proteins were identified between all three cohorts, with 57 proteins expressed after converted cHA seeding and 218 proteins shared between all three cohorts.

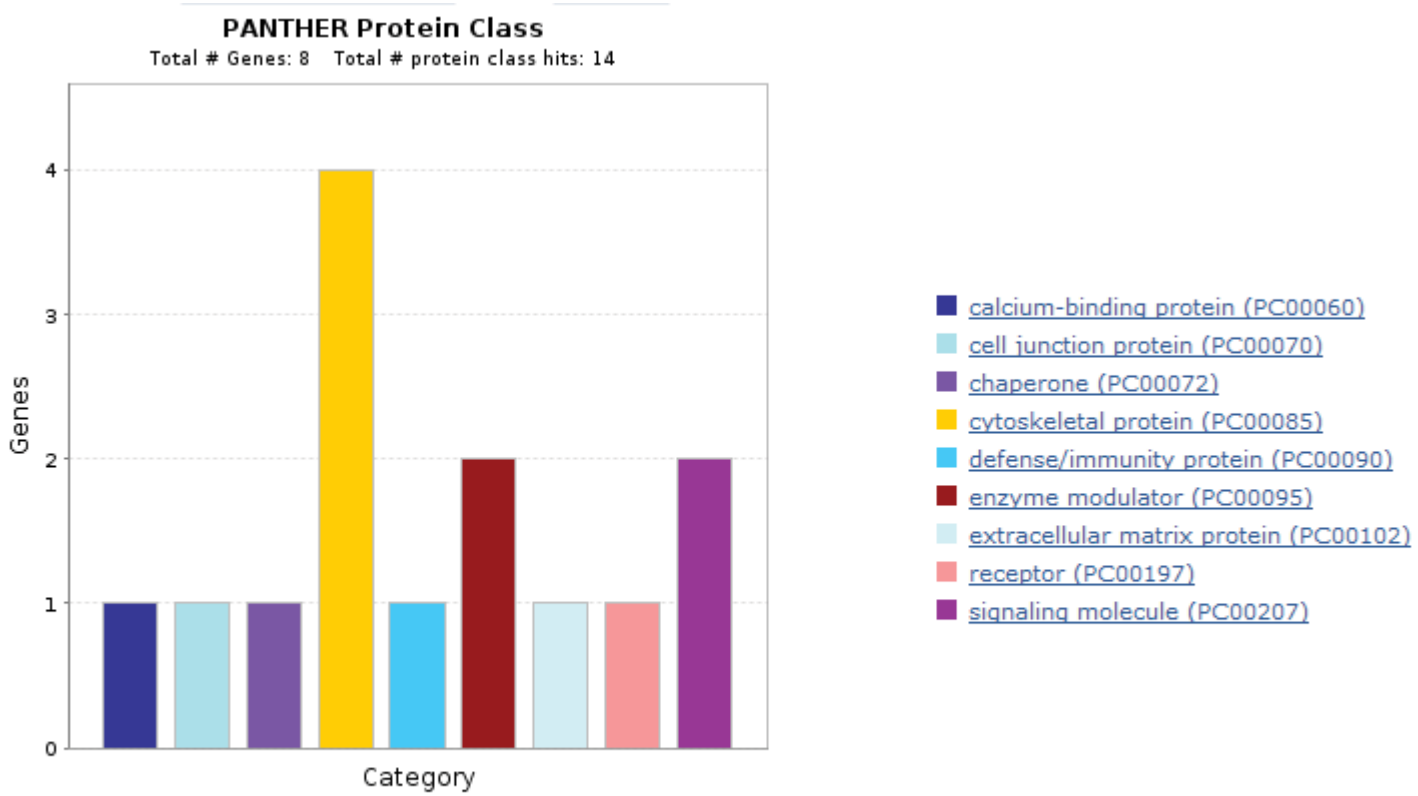

Figure 4.10: The annotated GO molecular function of proteins expressed after high density cHA treatment with the number of annotated genes on the y-axis and categorising molecular function on the x-axis with the legend corresponding in colour. It can be seen that a large number of proteins are annotated to be involved binding.

Table 4.2 Listed are the unique proteins identified from seeded converted cHA that are involved in calcium absorption. The proteins are sorted with their respective TrEMBL /Uniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Protein Score	Coverage(%)	Role in Bone Morphogenesis
P02545	Lamin A/C	57	16.3	Lamin A/C, also known LMNA, is a component of the lamina and is required for the normal development of the skeletal system as it plays an important role in nuclear assembly during osteoblastogenesis and bone formation [352].
Q00610	Clathrin heavy chain 1	194	6.9	Clathrin is a trimer of heavy chains that mediate endocytosis for membrane protein trafficking. It is involved in the uptake of ligand receptor complexes, membrane transporters and adhesion molecules [353].
Q8WXE9	Stonin-2	60	3.8	Stonin 2 is an endocytic protein that interacts with the clathrin/AP-2 complex as an AP-2 dependent sorting adaptor protein which targets synaptotagmins for endocytic internalisation[354]. Synaptotagmins enable calcium-induced exocytosis of essential ECM molecules in differentiating osteoblasts[355].

O96019	Actin-like protein 6A (ACTL6A)	841	54.9	ACTL6A alters the DNA nucleosome topology to suppress selective genes and is also involved in transcriptional activity [356]. Additionally, ACTL6A was found be expressed by somatic adult precursor cells to control nucleosome associated differentiation [357].
Q9Y696	Chloride intracellular channel protein 4	118	20.2	Chloride intracellular channel proteins are expressed during hypercalcemic events which generally occur at bones and joints in the body [358]
Q9UQM7	Calcium/calmodulin-dependent protein kinase II (CamKII)	53	5.1	CamKII is one of a family of calcium/calmodulin dependent protein kinases that play a central role in the transduction of calcium signals within the cell. During mechanical stimulation, CamKII is found to be expressed in normal osteo-articular tissue [359]`.

Table 4.3 Listed are the unique proteins identified from seeded unconverted cHA that are involved in calcium binding. The proteins are sorted with their respective TrEMBL /Uniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Protein Score	Coverage(%)	Role in Bone Morphogenesis
Q9BSJ8	Extended synaptotagmin-1	73	3.6	Synaptotagmin-5 mediates calcium-dependent binding at synaptic vesicles for calcium-induced exocytosis of essential ECM molecules in differentiating osteoblasts [311].
P08123	Collagen alpha-2(I) chain (ColA2)	63	2.6	Collagen alpha 2 combines with pro alpha 1 chain to form type 1 procollagen. This subsequently forms bone collagen fibers that get mineralised by calcium hydroxyapatite [360].
P26038	Moesin	60	9.7	Moesin is a member of the ERM protein family which acts as a cross linker between the plasma membrane and the cytoskeleton of a cell which allows cell to cell signaling. Moesin and CD44 are known to form a protein complex within osteoclasts. This CD44-moesin-actin filament system allows the osteoclasts to recognize the bone surface and initiate bone resorption [361] [362].
P11717	Cation-independent mannose-6-phosphate receptor	59	2	Also known as insulin like growth-factor 2 receptor, this protein regulates ECM deposition in osteoblast like cells by conversely inhibiting insulin growth-factor 1 [363].

Table 4.4 Listed are the unique proteins identified from seeded macroporous cHA that are involved in actin production and folding.

The proteins are sorted with their respective TrEMBL /Uniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis

Accession	Protein Name	Protein Score	Coverage (%)	Role in Bone Morphogenesis
P59998	Actin-related protein 2/3 complex subunit 4 (Arp 2/3 complex)	46	14.3	Arp 2/3 complex protein activates actin filaments to maintain multiple forms of actin based structures that occur during the constant rearrangement of cytoskeletal framework of cells during cellular differentiation and migration[364] [365].
Q16658	Fascin homolog 1, actin-bundling protein	107	18.5	Fascin is an actin binding protein which colocalises actin filaments to the periphery of the cell to meditate cell adhesion [366]
P60981	Destrin	75	20	Destrin regulates disassembly and assembly of actin filaments which are essential for cellular membrane ruffling and extension, a process common in osteoblast differentiation into osteocytes during bone development[322].
P40227	T-complex protein 1 subunit zeta	68	17.1	A molecular chaperone that assists in the folding of actin and tubulin *in vitro*[367], The proper folding of actin and tubulin is essential for many biological processes however its role in osteogenesis has not been elucidated.
P11047	Laminin subunit gamma-1 (Lγ-1)	100	5.9	Lγ-1 binds to skeletal ECM components with high affinity to mediate attachment and organisation of neighboring cells. Additionally, Lγ-1, Lα-1 and Lβ-1 has been found to be differentially expressed during osteogenesis [368]

P06703	Protein S100-A6	65	8.9	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell adhesion and differentiation [369]. Protein S100-A6, also known as calcyclin is responsible for sensing extracellular calcium and other cations such as aluminum and magnesium to regulate the osteoblast function [370].

Table 4.5: Listed are the unique proteins identified from seeded nanoporous cHA that are involved in calcium binding proteins. The proteins are sorted with their respective TrEMBL /Uniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis

Accession	Protein Name	Protein Score	Coverage(%)	Role in Bone Morphogenesis
P26447	Protein S100-A4	64	8.9	S100A4, also known as calvasculin, a calcium binding protein that interacts with microtubilin, actin and myosin to regulate cytoskeletal dynamics and elements such as cellular proliferation[371]. Interestingly, S100A4 has known to upregulate MMP-13 to stimulate chondrocyte differentiation [372].
P06703	Protein S100-A6 (Calcyclin)	63	8.9	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell adhesion and differentiation [369]. Protein S100-A6, also known as calcyclin is responsible for sensing extracellular calcium and other cations such as aluminum and magnesium to regulate the osteoblast function [370].
Q15149	Plectin	57	3.8	Plectin is a cytoskeletal cross linking protein which maintains mechanical integrity of many skeletal tissues, including muscle and bone. Additionally, the inhibition plectin has shown to bypass cellular senescence in rat fibroblasts [373].

Q02818	Nucleobindin-1	91	8.2	Nucleobindin 1 is a multifunctional calcium binding protein that was identified in the ECM of bone matrix and was later termed calnuc [374]. Within the bone matrix, calnuc binds and deposits calcium to form hard tissue. Additionally, calnuc binds hydroxyapatite to import calcium inside osteoblasts and osteocytes [375].
P27824	Calnexin	86	16.6	Calnexin is a calcium binding protein commonly expressed within the microvesicles of osteoblast like cells, called Saos-2 cells. It's thought that calnexin is an important component in the mineralisation process of HA within the lumen of differentiating Saos-2 cells [324].

Table 4.6 Listed are the unique proteins identified from seeded high density cHA that are involved in osteo-regulation. The proteins are sorted with their respective TrEMBL /Uniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis

Accession	Protein Name	Protein Score	Coverage (%)	Role in Bone Morphogenesis
O43776	Asparagine--tRNA ligase, cytoplasmic (NARS)			NARS is responsible for the attachment of asparagine to tRNA during the process of protein translation and is heavily involved in N-linked glycosylation of proteins. Furthermore, Park *et al* was able to show NARS to be crucial for the survival of FGF-2 induced osteoblasts [41].
P17844	Probable ATP-dependent RNA helicase DDX5	95	9.3	DDX5 is a multifunctional DEAD box protein that regulates gene expression by affecting RNA processing and transcriptional initiation. DDX5 enhances Runx3 transcriptional activity and thus increases osteoblast differentiation [376].
P09525	Annexin A4	133	20.4	Annexins are well-characterized in bone as components of mineralizing matrix vesicles[300]. Annexin A4 is a calcium dependent phospholipid membrane binding protein modulates NF-κβ signalling by directly interacting with the p50 heterodimeric p50/65 NF-κβ complex which participates in the RANKL pathway of osteoclast regulation [301] [302].

Q02818	Nucleobindin-1	91	8.2	Nucleobindin 1 is a multifunctional calcium binding protein that was identified in the ECM of bone matrix and was later termed calnuc [374]. Within the bone matrix, calnuc binds and deposits calcium to form hard tissue. Additionally, calnuc binds hydroxyapatite to import calcium inside osteoblasts and osteocytes [375].

Table 4.7 Listed are the unique proteins identified from seeded high density cHA that are involved in structural integrity protein and extracellular binding. *The proteins are sorted with their respective TrEMBL /Uniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis*

Accession	Protein Name	Protein Score	Coverage(%)	Role in Bone Morphogenesis
P20700	Lamin B1, isoform	116	25	Lamin B1 is a multifunctional nuclear intermediate filament that supports the structural integrity of the nucleus, and play a key role in stem cell niche function such as cell proliferation and differentiation of specific lineages, including bone formation [377]. A high ratio of lamin B is correlative to the stiffness of a cell and thus bone tissue is highly abundant in lamin A and lamin B [225].
Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	59	6.6	Procollagen-lysine,2-oxoglutarate 5-dioxygenase (PLOD-3) post transitionally modifies collagen through hydroxylation of lysine residues leading to the binding and cross-linking of fibrils to collagen fibers. This provides tensile strength to collagen fibers within the organic matrix of bone[378].
Q5T1M5	FK506-binding protein (FKBP)	54	7.8	FKBP is a protein that has prolyl isomerase activity that coordinates cytoskeletal organisation through the transport of early endosomes between microfilaments. Mutations within the FKBP gene leads to development of osteogenesis imperfecta as the proper folding of the collagen triple helix does not occur for procollagen 1 secretion during skeletal development [379].

O75369	Filamin-B	191	6.1	Filamin B cross-links actin which enables intracellular communication between the cytoskeleton and cell membrane during skeletal development. Various studies have shown that a mutation within the filamin B gene can lead to an impairment of bone formation [380]

Table 4.8 Listed are the unique proteins identified from seeded converted cHA that are involved in osteoclastogenesis and bone resorption. The proteins are sorted with their respective TrEMBL /Uniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis

Accession	Protein Name	Protein Score	Coverage(%)	Role in Bone Morphogenesis
Q9Y490	Talin-1	60	3.7	Talin-1maintains the actin cytoskeleton adhesion by complexing with various integrins. During osteoclast activation,Talin-1 binds to macrophage colony stimulating factor (MCSF) and β integrin to mediate proper cytoskeleton rearrangement to ensure osteoclast viability [381].
P21810	Biglycan	71	10.4	Biglycan extracellular proteoglycan that positively regulates bone formation and osteoclast differentiation through its effects on osteoblasts and their precursors [382].
K7EJ44	Profilin 1	328	29.2	Profilin- 1 is a ubiquitously expressed G-actin protein that promotes actin polymerisation. It is essential for actin reorganization during osteoclast bone resorption [383]. Additionally, profilin is highly upregulated during BMSCs osteodifferentiation [303].
P26038	Moesin	60	9.7	Moesin is a member of the ERM protein family which acts as a cross linker between the plasma membrane and the cytoskeleton of a cell which allows cell to cell signaling. Moesin and CD44 are known to form a protein complex within osteoclasts. This CD44-moesin-actin filament system allows the osteoclasts to recognize the bone surface and initiate bone resorption [361,362].

P00918	Carbonic anhydrase 2	69	12.9	Carbonic anhydrase II is a cytoplasmic enzyme highly abundant in osteoclasts as they are essential for bone resorption and osteoclast differentiation in vivo and in vitro [384] . Interestingly, their osteoclastic effect is heavily mediated by anti-inflammatory factors [385].
O75955	Flotillin-1	67	9.3	Flotillin-1 is a membrane protein that has been distributed exclusively in a lipid raft membrane fractions in various cells. During osteoclastogneogenesis, the raft component flotillin are increased after stimulation with macrophage colony stimulating factor [293].
P21333	Filamin-A (FLNA)	687	14.8	FLNA is an actin binding and cross linking protein which regulates the cytoskeletal network to regulate signal transduction during cell migration [386]. Filamin A is required for osteoclastogenesis as FLNA null progenitor cells cannot undergo osteoclastogenesis *in vivo* and *in vitro*. Furthermore, FLNA null animal models show skeletal abnormalities and skeletal dysplasia [387].
B7Z992	FLJ53698, highly similar to Gelsolin	124	10.7	Isoforms of gelsolin are an actin regulating protein for osteoclast precursor cells [241]. During osteoclast maturation, osteopontin regulates the interaction of gelsolin to form cytoskeletal rings important for osteoclast formation during bone remodeling [298].
P35749	Myosin-11	429	17.9	Myosin 11 participates in the stabilisation of osteoclast lamellipodium which allows the osteoclasts to resorb substrate within the lacunae of bone [388].

Q14213	Interleukin-27 subunit beta	45	3	27 is cytokine that is structurally related to IL-2 and IL-23. Molecular entities such as p68 are essential for formation of functional IL-27[389]. Studies have shown that IL-27 plays a major role in the regulation of osteoclastogenesis as it affects both RANKL dependent and independent pathway [390]s.
K7EJ44	Profilin 1	328	29.2	Profilin- 1 is a ubiquitously expressed G-actin protein that promotes actin polymerisation. It is essential for actin reorganization during osteoclast bone resorption [383]. Additionally, profilin is highly upregulated during BMSCs osteodifferentiation [303].
Q07020	ribosomal protein L18, isoform (RPL18)	74	11.7	RPL18 is a ribosomal protein that is a component of the 60S ribosomal subunit. RPL18 has shown to bind double-stranded RNA-Activated Protein Kinase (PKR) to inhibit PKR-dependent eIF-2a phosphorylation and consequently stimulate translation initiation of osteoclastogenesis [391,392].

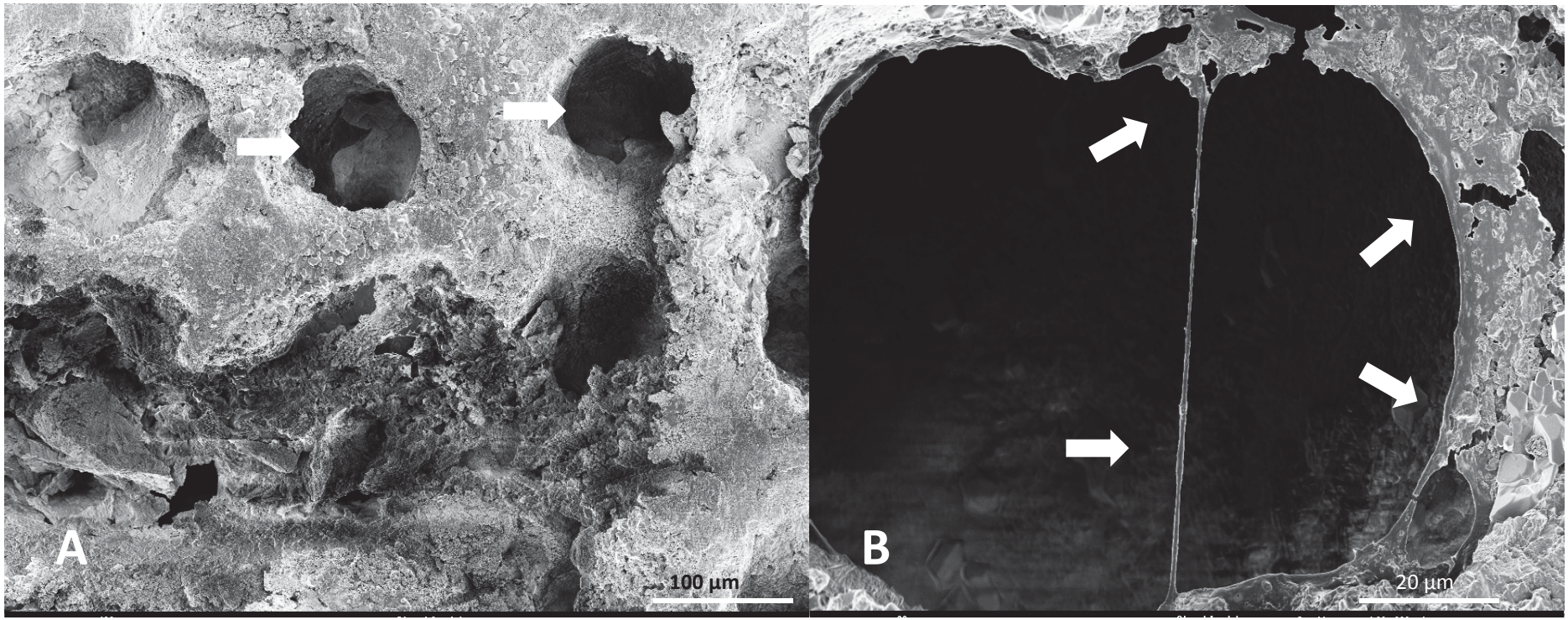

Figure 4.11 Scanning electron micrographs (SEM) showing the surface structure and cross-sections of High Density cHA. (S) Unseeded after 30 days in hADSC conditioned medium, the interconnected pores are empty (white arrows) with no observable growth of cells; (B) Seeded image showing cells proliferating across the porous HA (white arrows).

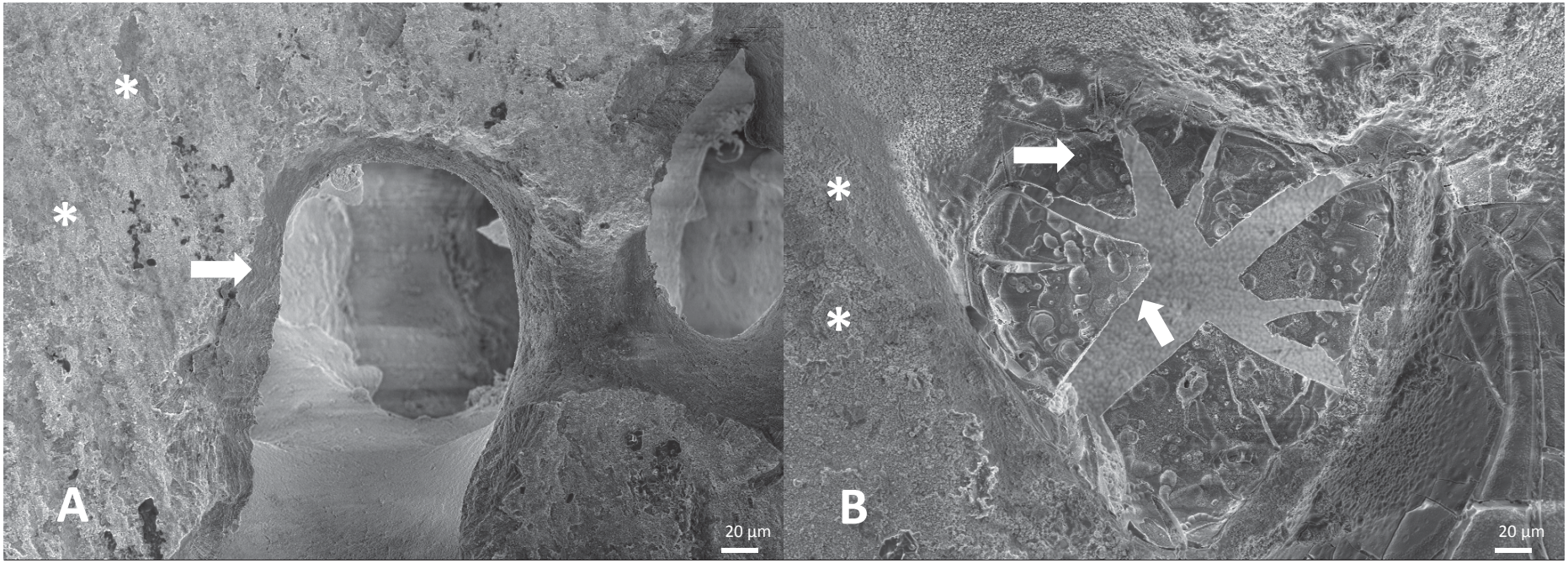

Figure 4.12 Scanning electron micrographs (SEM) showing the surface structure and cross-sections of nanoporous cHA. (A) Unseeded for 30 days in hADSC medium, showing the absence of cellular growth over the interconnected pores (white arrow); (B) Seeded image showing a fragmented monolayer of cells across the porous HA (white arrow). The flat regions of the nanoporous cHA have no signs of nano porosity (white asterisk) indicating successful nano coating.

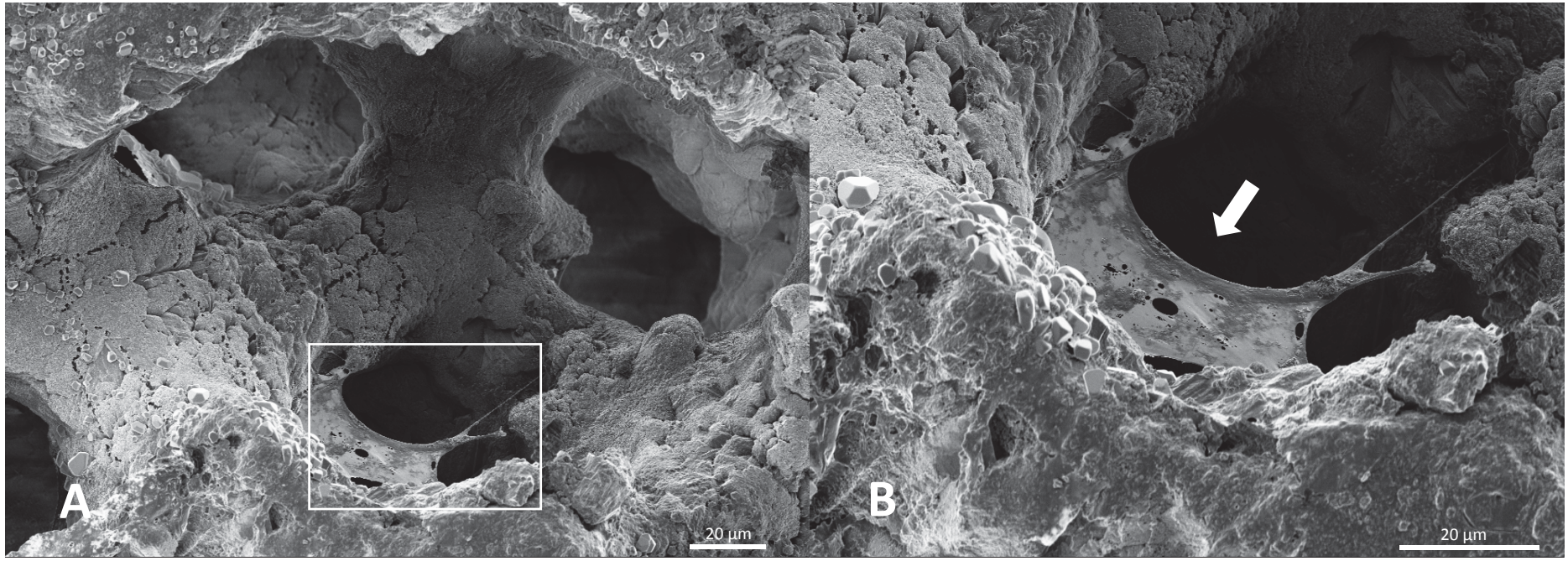

Figure 4.13 Scanning electron micrographs (SEM) at different magnifications showing the surface structure and cross-sections of Converted cHA. (A) at a magnification of 500 X showing the consistency of hADSC seeding after 30 days (white box) ; (B) 1.00 K magnification of the region highlighted by the red box in image A, showing a monolayer of cells proliferating across a small portion of the porous HA.

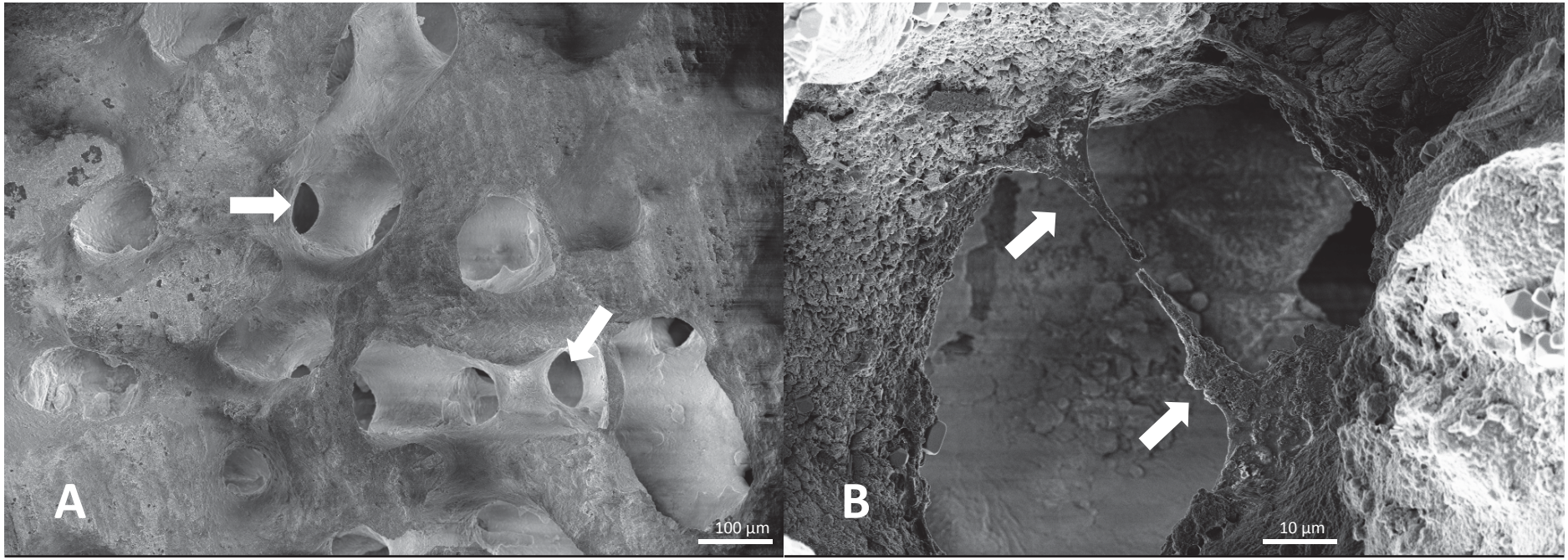

Figure 4.14 Scanning electron micrographs (SEM) showing the surface structure of cross-sections unconverted coralline calcium carbonate. (A) Unseeded after 30 days in hADSC medium, showing zero proliferation over the porous structure (white arrows); (B) 30 Days Seeded showing a thin microfilament of cells proliferating across the porous HA that appear granulated (white arrows).

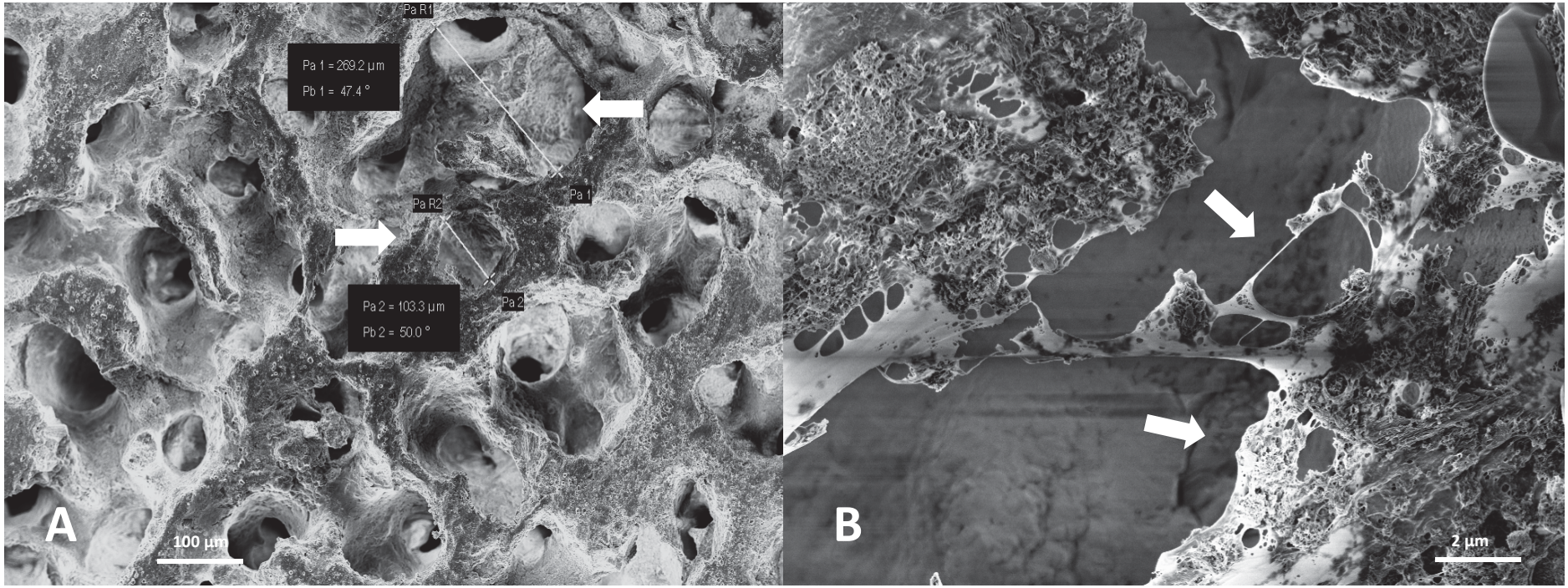

Figure 4.15 Scanning electron micrographs (SEM) showing the surface structure of cross-sections macroporous cHA. (A) Unseeded after 30 days in hADSC medium, showing zero proliferation over the porous structure (white arrows) and the large porous architecture of the scaffold (white lines); (B) 30 Days Seeded showing a thin microfilament of cells proliferating only on the surface of macroporous cHA that appear granulated (white arrows).

Table 4.9: The secretion profile of seeded converted cHA reported as either as high or moderate in concentration and sorted by their reported role within the literature. '*' represents a cytokines dual functionality.

Converted cHA	Pro-inflammatory	Anti-inflammatory	Angiogenesis	Fibroblast recruitment
High	Il-6*	IL-1ra	---	FGF-basic
	IL-8	Il-6*	---	---
	IL-17A	---	---	---
Moderate	---	---	VEGF	MCP-1
	---	---	---	G-CSF

Table 4.10: The secretion profile of seeded unconverted cHA reported as either as high or moderate in concentration and sorted by their reported role within the literature. '*' represents a cytokines dual functionality.

Unconverted cHA	Pro-inflammatory	Anti-inflammatory	Angiogenesis	Fibroblast recruitment
High	IL-6*	---	---	---
	Il-8	IL-6*	---	---
Moderate	IFN-y	IL-1ra	VEGF	FGF Basic
	---	---	---	GCSF

Table 4.11: The secretion profile of seeded nanoporous cHA reported as either as high or moderate in concentration and sorted by their reported role within the literature*. '*' represents a cytokines dual functionality.*

Nanoporous cHA	Pro-inflammatory	Anti-inflammatory	Angiogenesis	Fibroblast recruitment
High	IL-6*	IL-Ira in nano	VEGF in nano	G-CSF
	IL-8	IL-6	---	---
	TNF-alpha	---	---	---
	IL-1	---	---	---
Moderate	IL-17A	---	---	GM-CSF
	IFN-y	---	---	MCP-1

Table 4.12: The secretion profile of seeded macroporous cHA reported as either as high or moderate in concentration and sorted by their reported role within the literature*. '*' represents a cytokines dual functionality*.

Macroporous cHA	Pro-inflammatory	Anti-inflammatory	Angiogenesis	Fibroblast recruitment
High	IL-6	---	VEGF	G-CSF
	Il-8	---	---	---
	IL-1ra	---	---	---
Moderate	IL-17A	---	---	MCP-1
	IFN-y	---	---	---

Table 4.13: The secretion profile of seeded High Density cHA reported as either as high or moderate in concentration and sorted by their reported role within the literature. '*' represents a cytokines dual functionality.

High density	Pro-inflammatory	Anti-inflammatory	Angiogenesis	Fibroblast recruitment
High	Il-6	---	---	GM-CSF
	IL-8	---	---	G-CSF
Moderate	RANTES	---	VEGF	---

4.5 Discussion

ADSCs are considered to be highly useful for tissue repair due to their multipotent differentiation potential. However, there is emerging evidence that MSCs coated onto coralline scaffolds can influence bone regeneration *in vivo* [393] and *in vitro* [394]. Most studies evaluate the osteogenic nature of coralline hydroxyapatite through histological and gene expression analysis of either the defect site [395] or the MSCs seeded onto the scaffold itself [264]. In this study we have used discovery proteomics to further understand the molecular mechanism behind the osteoconductive nature of coralline scaffolds. Furthermore, coralline scaffolds can be augmented or fabricated from different coral genera, each having a unique property that could be harnessed for bone tissue engineering.

Therefore, the identification of hADSC proteins seeded onto different coralline scaffolds can allow us to understand if, hydrothermally converted cHA, nanoporous cHA, macroporous cHA and high density cHA have effects on the proteome of hADSCs. This was examined using a shotgun proteomic analysis using LC-MS/MS which allowed us to identify a change in the hADSC protein profile after seeding onto different coralline scaffolds (Table 4.1). An additional 397 unique proteins were identified from hADSCs post coralline scaffold seeding, from which over 250 osteogenic proteins were identified between the five different types of coralline material.

A study assessing the effectiveness of porites coral against beta-tricalcium phosphate (β-TCP) and a bone allograft consisting of morcelised human corticocancellous bone found osteogenesis to occur more efficiently over MSC seeded porites coral than the other biomaterials tested *in vitro* and in animal models [396]. Furthermore, the study showed that new bone formation does not occur if the coralline scaffolds are not seeded with MSCs, highlighting the importance of coating biologically relevant cells onto potential scaffold constructs. However, the molecular reason for the effectiveness of coralline material was not elucidated and instead the authors attributed the osteo-viability of porites coral to the porous architecture of the scaffold.

Attempts have been made to investigate the osteoconductive nature of HA. A proteomic study investigating the effect of natural hydroxyapatite (nHA) seeded with BMSCs identified a number of molecular pathways potentially involved in nHA induced osteogenesis of MSCs such as the MAPK signalling pathway, the TGF-beta receptor signalling pathway, the adipogenesis pathway, the actin cytoskeleton regulatory pathway and the epidermal growth factor receptor (ErbB) signalling pathway [397]. However, within the previously mentioned study, only the Mitogen-activated protein kinases (MapK) pathway was confirmed to induce osteogenesis experimentally as the inhibition of the MapK associated mRNA was found to affect the induction of osteogenesis.

Different osteogenic signalling pathways have been linked to hydroxyapatite induced osteogenesis in MSCs. This was recently revealed by a study which modified the surface chemistry of hydroxyapatite via the addition of nano-sheet modifications. This allowed rat BMSCs to undergo and maintain osteogenesis *in vitro* [398]. The study associated the ERK pathway to induce osteogenesis. Furthermore, nano-sheet and nano rod surface augmentation of hydroxyapatite induced two different molecular pathways (ERK and MapK) for bone morphogenesis *in vitro*. Moreover, it has also been shown that pore interconnectivity has a direct effect on increasing vascularisation of seeded progenitor cells [399].

This leads to the premise that coralline scaffolds with altered surface chemistry similar to nano coating or a variation in porosity, can control fundamental cellular behaviour such as proliferation, migration, differentiation, and consequently *in vivo* osteo-integration. Similarly, we investigated the effect of high density, macroporous, nanoporous, converted and unconverted coralline scaffolds on the hADSCs phenotype where a shift in the proteomic, chemokine and cytokine signature after seeding was identified. In particular, seeding a nano coated nanoporous cHA allowed hADSCs to express 30 different proteins in comparison to our basal hADSCs control cohort. Within this dataset, up to 15 different osteogenic proteins were identified and the bioplex assay revealed different levels of cytokine expression. In particular, high expression of

IL-1ra, G-CSF and VEGF were unique to the seeded nanoporous secretion profile. The chemokine VEGF is a vascular endothelial growth factor that mediates bone growth via the endochondral ossification pathway [400] as it mediates the vital phase of vascularisation and angiogenesis to occur at the fracture site during bone repair [401]. Interestingly, proteins involved in vascularisation and wound repair such as anti-thrombin III and collagen I were identified in our seeded nanoporous hADSC protein profile. Antithrombin III inhibits the downstream coagulation pathway of blood clotting through the inactivation of the essential coagulation protein thrombin (and Factor Xa). This process actively plays a role in tissue repair during the initial formation of a hematoma [214]. Furthermore, our SEM images showed proliferation of hADSCs throughout the nano porous architecture of scaffold. This indicates that attachment and cellular proliferation did occur after the seeding process, however an animal model would be required to confirm vascularisation.

Additionally, IL-1ra is an immunomodulatory cytokine which antagonises the inflammatory effect of IL-1 [402]. The high expression of IL-1ra in our seeded nanoporous cohort indicates that nano coating coralline cHA may induce an anti-inflammatory effect on the hADSCs. The anti-inflammatory response could be due to the high prevalence of lL-6 and IL-8 which were also detected in our nanoporous secreted profile. The cytokine IL-6 acts both as a pro and anti-inflammatory mediator [403]. To mediate an anti-inflammatory effect, IL-6 initially activates IL-1ra while concurrently inhibiting IL-1, IL-10 and TNF-α [404]. Therefore, the overexpression of IL-1ra and IL-6 may be a response to control the inflammatory microenvironment of the seeded nanoporous cHA.

This is further supported through our proteomic identification of the anti-inflammatory protein IKBKB (Inhibitor of nuclear factor kappa-B kinase interacting protein). This protein inhibits the NF-κB pathway which is responsible for the inflammatory loss of bone mass during osteoporosis [405]. The importance of inhibiting the NF-κB was highlighted by Chang *et al* who showed *in vivo* and *in vitro* inhibition of NF-κB maintains bone mass and matrix formation within osteoblasts [406]. The molecular

reason for such an inflammatory microenvironment within the nanoporous cHA is yet to be established. Additionally, the presence of fibroblastic proteins from our proteomic profiling of seeded nanoporous cHA suggests a subset of proteins may be involved in intracellular fibroblast recruitment. As previously mentioned in chapter 1, an aggregation of fibroblasts and clotting factors is the initial stage of *in vivo* bone tissue repair. During these events, the fracture microenvironment maintains a highly inflammatory response as part of a downstream coagulation pathway to initiate bone healing [30]. Additionally, the detection of high concentrations of the chemokine G-CSF and the detection of LRRC 59, a nuclear transporting protein which exclusively imports fibroblast growth factor 1 (FGF1) [407] suggests that seeded nanoporous cHA may promote the initial stages of bone regeneration.

Within the unconverted coralline calcium carbonate scaffold protein dataset, a total of 160 proteins were identified, from which only 13 proteins were found to be uniquely associated with unconverted coralline material. These unique proteins were 60S ribosomal protein L23, Protein S100-A9, integrin alpha-V (IαV2) isoform 2 precursor (IαV2), Protein S100-A8, Ribosomal protein S6 and Transgelin 2. The GO annotation revealed these proteins to be involved in cell adhesion and structural integrity. This analysis was further supported by our literature review which identified the family of S100 proteins A8 and A9 to be involved in calcium binding and regulation of cell migration and adhesion [408]. Additionally, the S100-A8/S100A9 complex initiates the NFκB and MAP kinase signalling which as previously mentioned, has been shown to promote osteogenesis in MSCs [409,410].

The presence of the aforementioned osteogenic regulatory proteins found only in hADSCs seeded on unconverted coralline scaffolds may explain why aragonite can still be osteoconductive without having to undergo hydrothermal conversation [411]. However, a primate animal model study performed by Ripamonti *et al* highlights that osteoconductivity of coralline material increases after hydrothermal conversation [412]. This was also evident in our proteomic data which identified a higher cohort of osteogenic proteins in seeded converted cHA in comparison to unconverted coralline

material. The exact pathway or the extent to which osteogenesis has occurred in our study is yet to be determined. It is proposed that the BMP pathway may be involved as the downregulation of BMP-2 leads to the up regulation of noggin genes during bone repair in primates implanted with coral derived macroporous constructs [413].

Within our study, there was an absence of key osteogenic markers such as BMP, BMP2, osteocalcin and osteopontin. However, the literature survey of the proteins identified did reveal a number of osteoclast proteins from our multiple coralline cHA cohorts. More notably, converted cHA expressed annexin A4, flotillin-1, filamin A, gelsolin, talin-1, biglycan, profilin 1, moesin and carbonic anhydrase, all of which play a role in either regulating or initiating osteoclastogenesis (Supplementary Table 4.1 and Table 4.8). Additional osteoclast proteins were identified in our seeded macroporous cHA dataset with proteins such Myosin-11, Profilin 1, Interleukin-27 subunit beta, ribosomal protein L18, isoform (Table 4.8).

The presence of osteoclast proteins on seeded coralline scaffolds plays an important role in coral resorption. Coral resorption is required for efficient osteo-integration and bone apposition of coralline medical implants as evidenced by Guillemin *et al* [64]. Coral resorption allows the eventual replacement of coralline material with new bone during fracture repair. Guillemin and co-workers identified osteoclastic activity was the basis of coral resorption i*n vivo* and they credited this role to carbonic anhydrase, an enzyme detected in our seeded converted cHA protein profile. Furthermore, coral resorption is linked to pore interconnectivity [411] which may explain why our converted cHA expressed a larger repertoire of identified osteoclastic proteins in comparison to the other coralline scaffolds. The importance of calcium ions and osteoclast activity during bone morphogenesis was further demonstrated by *Klar et al* who performed an *in vivo* study that implanted converted cHA coated with a known calcium blocker (verapamil) and an osteoclast inhibitor (zoledronate) [414]. This coating prevented the induction of bone formation. Therefore, the identification of osteoclastic proteins suggests that coral resorption may be taking place on seeded converted cHA after 30 days.

Additionally, a large repertoire of cell adhesion and proliferation proteins were identified in our seeded macroporous coralline proteomic profile. Cellular adhesion and proliferation is vital for efficiently seeding an orthopaedic scaffold which can lead to vascularisation and ultimately regeneration of bone tissue within an implant material [415]. The effectiveness of coralline hydroxyapatite at permitting cellular attachment has been the focus of various studies. For example, Devecioğlu *et al* showed coralline hydroxyapatite has superior cellular attachment and proliferation in comparison to cryopreserved bone and demineralised dentin [416]. Additionally, Doherty *et al* demonstrated the attachment proficiency of commercial cHA Biocoral® which again showed superior cellular attachment when compared to commercial bone construct SurgiBone® and Ostolit® [417]. However, both studies concluded that the mechanism through which converted cHA provides a superior seeding efficiency is not well understood. Some studies have showed that within seconds of implantation *in vivo*, biomaterials are coated with an adsorbed layer of integrins that mediates progenitor cellular attachment for osteo-regeneration [418]. Specifically the integrins vitronectin and fibronectin [419], both of which were expressed by our seeded coralline hADSCs.

In conclusion, our study investigated the hADSCs seeded onto different coralline scaffolds and found different proteomic profile, each with a unique phenotype that plays a role in bone morphogenesis. We also detected a unique secretion profile of seeded nanoporous cHA which indicated a highly pro-inflammatory microenvironment with fibroblast recruitment potential, a phenotype commonly seen during the first stages of bone fracture repair. Additionally we compared the proteomic profile of seeded unconverted coralline calcium carbonate and converted cHA and identified a number of osteoclast regulatory proteins that may potentially play a role in coral resorption and ultimately vascularisation. Finally, we identified adhesion and proliferation proteins expressed after seeding onto macroporous cHA suggesting that similarly to converted cHA, macroporous cHA also has a greater efficiency at allowing cellular attachment and ultimately internal vascularisation.

4.5 Conclusion

Coralline materials can augmented to accommodate a range of desirable orthopaedic properties such as high mechanical strength, greater flexibility and more importantly, the ability to effectively promote bone regeneration as safely as possible. Here we proteomically characterised hADSCs seeded onto multiple coralline scaffolds such as macroporous cHA, nanoporous cHA, high density cHA, unconverted coralline carbonate and converted coralline HA. The unique proteomic signature of seeded macroporous cHA showed a large number of cellular adhesion and ECM proteins involved in mediating binding and regulating the cytoskeletal matrix which suggested that the large porous architecture of scaffold created a challenging microenvironment for the hADSCs to attach and proliferate. This was further supported with scarcity of hADSCs observed by SEM (Figure 4.15). The chemokine profile however showed seeded macroporous cHA promoting angiogenesis and a pro-inflammatory response to which we credited to the large porous architecture of the scaffold itself.

Our SEM observation of seeded nanoporous cHA demonstrated hADSCs can completely proliferate across the small porous architecture of the coralline scaffold. Here we show that the hADSCs exhibited an anti-inflammatory and a pro-inflammatory response to the nanoporous cHA with the proteomic signature showing the unique expression of proteins involved in facilitating downstream anti-inflammatory effects. Additionally, high concentrations of chemokines involved in angiogenesis and fibroblast recruitment was also detected suggesting that nanoporous cHA promoted a environment typically seen during the first couple of days in fracture repair. However the biological significance of this finding should be pursued in subsequent animal model. We also identified the proteomic response of hADSCs seeded onto high density cHA which consisted of structural integrity proteins and extracellular binding proteins in conjunction with the secretion profile revealing a high concentration of chemokines involved in fibroblast recruit. The biological significance of this finding is yet to be elucidated.

Here we also showed the effect of hydrothermal conversation in changing the bioactive properties of coralline scaffolds. Unconverted coralline carbonate reported a lower number of osteogenic associated proteins in comparison to converted cHA. Furthermore, the bioplex secretion profile also showed differences in chemokine expression. Notably, seeded converted cHA demonstrated a higher pro-inflammatory and angiogenic response similarly to unconverted cHA which reported a similar immunomodulatory response but at a lower concentration with different types of chemokines.

Together these findings highlighted that minor characteristic differences between the fabrications of coralline scaffolds can induce a detectable change in the phenotype of seeded hADSCs. Further work is aimed at reproducing these observed biological responses during fracture repair through an *in vivo* animal model

Chapter Five: Final Summary and Future Directions

The overarching aim of this thesis was to investigate the effect of coralline scaffolds on adipose derived stem cells (ADSCs) for bone tissue engineering. This has been achieved by initially seeding coralline hydroxyapatite (HA) with rat adipose derived stem cells (rADSCs) for 14 and 30 days and identifying the protein profile, and by implication the phenotype, using LC/MS/MS. Following this, we assessed cellular proliferation with scanning electron microscopy (SEM) and the indirect effect of HA on rADSCs *in vitro* with histological staining **(Aim 1)**. I then assessed the insoluble membrane subproteome of seeded rADSCs through ultracentrifugation isolation and subsequent solubilisation **(AIM 2)**. Finally, we changed our model organism from rADSCs to human adipose derived stem cells (hADSCs) and investigated the effect of multi coralline scaffolds (coralline carbonate, high density HA, nanoporous HA and macroporous HA) on hADSCs by identifying and comparing their proteomic profile with LC/MS/MS and detecting their cytokine and chemokine expression with a Bioplex assay while assessing cellular proliferation with SEM **(AIM 3).** Together these findings contribute a vast knowledge of coralline seeded ADSC biology and document the shift in the cellular proteome under the influence of different coralline materials that ultimately induce the expression of osteogenic associated proteins *in vitro*. The production of these osteogenic proteins was found to occur under a high pro-inflammatory microenvironment that is commonly observed *in vivo* during fracture repair [420-422]. This suggests ADSCs can mediate molecular downstream pathways generally involved in tissue repair without the influence of any external growth factors or chemical cues.

5.1 The proteomic profiling of seeded coralline HA with rADSCs

The first study reported in this thesis identified a change in the proteomic signature of rADSCs after their seeding onto coralline HA for 14 and 30 days. We compared this proteomic signature to isolated primary rat osteoblasts and basal rADSCs to identify similarities and differences between the three cohorts. There was little similarity between the proteins of rat primary osteoblasts and seeded HA rADSCs. Instead we identified a group of osteogenic regulatory proteins that play a role in bone resorption, extracellular matrix (ECM) deposition and cellular adherence.

The effect of bone resorption may possibly be a response to the abundance of calcium ions present in the microenvironment of coralline hydroxyapatite as evidenced by the ICP/MS analysis of the ADSC culture medium (Section 2.3.3 and Figure 2.6) This ADSC biological response to extracellular factors released by coralline scaffolds is one of the advantages of utilising cellular material with orthopaedic constructs; however, this concept is not always applicable. For example, in a study by Gelberman and colleagues [423], autologous canine ADSCs (cADSCs) were able to effectively differentiate into osteo tissue *in vitro* under the effect of bone morphogenic protein 2 (BMP2) however the *in vivo* study of cADSCs seeded into a Heparin/fibrin-based delivery system (HBDS)/nanofiber scaffold was unsuccessful as the implant induced an inflammatory response and showed reduced regeneration [423]. The study attributed this detrimental effect of tendon degeneration to the HBDS/nanofiber scaffold system as it was shown to induce the expression of proteins associated with inflammatory cytokines, immunologic responses and matrix degradation. In contrast, the proteomic signature of cells seeded onto HA was different due to the expression of serine protease inhibitors such as vitronectin and Alpha-2 antiplasmin which limit the catalytic degradation of the ECM by serine proteases[424]. This highlights the importance of identifying the optimal biomaterial best suited for tissue regeneration as some seeded orthopaedic scaffolds can be detrimental to their respective target sites.

Additionally, only a few studies have utilised ADSCs seeded onto coralline scaffolds successfully. Cui *et al* differentiated ADSCs to osteocytes before seeding them into coralline carbonate in a cranial bone defect canine model[283]. Osteo-induction of ADSCs before seeding and implantation is advantageous as it commits the progenitor cells towards an osteogenic lineage however, as Cui and co-workers highlighted, there was no difference in ALP and osteocalcin expression between osteo-induced and non-induced seeded ADSC cohorts. This finding is shared among other studies [425] with Birk *et al* [426] reporting that ADSCs seeded onto a Porites coralline scaffold began differentiating into bone-like cells, similarly to our results.

Following this my work showed the indirect effect of HA on rADSCs where I confirmed the presence of calcium deposition commonly observed in osteo-differentiated MSCs[427]. I subsequently confirmed that HA in ADSC culture medium does steadily release calcium ions over 30 days (Figure 2.6). Similar to our findings, studies have demonstrated that calcium ions can induce a phenotypic shift in ADSCs where mineralised nodules of calcium are observed [428]. Interestingly, X-ray diffraction studies have characterised the mineralised nodule to have a crystalline structure characteristic of hydroxyapatite[265]. The capability to differentiate ADSCs with a single chemical ion is promising. However, the molecular mechanism through which calcium induces this phenotypic change in ADSCs was not the focus of our study and should be of interest for future investigation.

I have demonstrated that rADSCs undergo a change in their proteomic profile after seeding onto coralline HA. However, the number of proteins identified in the study could be considered low, given the biological context that there are over 19000 genes within the rat genome [429]. Factors such as ion suppression from high abundant proteins and limitations associated with instrument dynamic range can decrease the number of proteins identified by LC/MS/MS tandem mass spectrometry (MS). Ion suppression generally occurs in complex mixtures of peptides where high abundant proteins generate more observed peptides which can ‘mask’ any plausible peptides which share the same chromatographic retention time [430]. Additionally, the issue of

dynamic range does play a role as proteins with a low copy number may fall outside the practical detection limits of MS analysis [161].

There are solutions, such as additional peptide fractionation steps which can separate complex mixtures; and increasing sample concentration which increases the molecular concentration of proteins with low copy numbers, allowing detection within the limits of MS analysis. The aforementioned techniques should be utilised for all my treatments to increase the number of proteins identified. This would contribute towards a greater understanding of the proteomic changes taking place under the influence of seeded HA. We have partially attempted to identify a larger array of proteins in our study by analysing the 'subproteome' of membrane proteins which are proteins that would not be normally detected in our standard shotgun MS procedure.

5.2 The effect of coralline HA on the rADSC subproteome

In chapter 3, I first assessed three different methodologies that would best identify a wider array of proteins not detectable by our standard shotgun proteomic procedure. We demonstrated that trypsin digestion of the rADSC cell surface to release surface proteins can be performed for up to 15 minutes without the risk of cytotoxicity. Similar studies utilise this methodology for a fast proteomic identification of surface molecules however no known attempts have been made to replicate this process on the stem cell surface of rADSCs [326].

Our second methodology involved the labelling of rADSC surface proteins with sulfo-NHS biotin where I identified twice as many proteins than the surface trypsin digestion procedure. However, our Western blot of biotin labelled surface proteins revealed the possibility of cytosolic contaminants within our elutions and that our recovery of biotinylated proteins was insufficient. However, similar studies that have employed biotin labelling of membrane proteins were successful. Niehage and colleagues [325] identified over 200 *bona fide* plasma membrane proteins from bone marrow stem cells (BMSCs). A major difference in the biotin labelling protocol of Niehage *et al* and this work is the length of time applied for *in vitro* incubation with EZ-link sulfo NHS biotin. Our protocol labels surface proteins with biotin for 30 seconds, in comparison to Niehage and colleagues who allowed up to 1 hour for this bio labelling process. This additional contact time between the rADSC and sulfo NHS biotin may have allowed a higher number of surface proteins to be labelled and subsequently identified.

Following this we investigated the 'insoluble' fraction obtained during our shotgun proteomic analysis of rADSCs through ultracentrifugation and membrane solubilisation (UC-isolation). This approach yielded the highest number of membrane protein identifications that were previously not identified by any of our previously mentioned methodologies. We subsequently employed this technique for all our subsequent subproteome investigations of seeded HA membrane proteins. In total, UC-isolation allowed us to identify 179 proteins in our subproteome, and a subsequent literature

review and GO annotation revealed a number of calcium binding proteins, CD markers and ECM proteins that are involved in osteo-regulation. The aforementioned proteins have not been previously identified by our shotgun proteomic approach. Interestingly, the identification of the calcium binding proteins Calnexin and Calreticulin allowed us to identify the Wnt signalling pathway as a possible mediator of inducing the proteomic and phenotype change observed in rADSCs [431].

Despite all these efforts, the representation of membrane proteins in the aforementioned studies are scarce, within context; at least 27% of the human genome encodes for membrane proteins [432]. The difficulty in the identification of membrane proteins arises from their physiochemical properties such as hydrophobicity, which impedes the investigators ability to efficiently solubilise and separate these proteins from cytosolic contaminants. We attempted to overcome this challenge through the use of organic solvents, detergents, and non-specific peptidases to improve membrane protein solubility [433]. However, complete solubilisation of the membrane proteome from the cytosolic fraction continued to be difficult.

Furthermore, membrane proteins are less abundant than cytosolic proteins in mammalian cells and often possess extensive post-translational modifications [434], making multiple abundant identifications a difficult endeavour. Future work should continue to fractionate and solubilise the insoluble fraction to obtain a wider array of identified proteins. Recently, a high throughput membrane solubilisation screening assay published by Lantez *et al* [435] identifies the optimal solubilisation conditions suitable for most membrane investigations by testing 96 different solubilisation parameters in a single 96 well assay.

In summary, the knowledge built from this work sets precedence for an animal model to be adopted where the effectiveness of autologous ADSCs seeded on HA can be assessed for its bone repairing potential.

5.3 The proteomic profiling of coralline carbonate, converted cHA, high density cHA, macroporous cHA and nanoporous cHA seeded with hADSCs.

With the emergence of coralline material as a potential bone substitute, there have been many augmentations made by researchers to the material to improve its mechanical and biological properties [187,256,346]. Multiple studies have noted a phenotypic change that is characteristic to bone cells in progenitor cells seeded onto coralline scaffolds yet only a few have investigated the molecular reasoning behind this phenomenon.

Multiple coralline scaffolds were seeded (Table 4.1) and their proteomic changes and cytokine and chemokine secretion profiles after 30 days investigated. It was evident that all forms of coralline material did induce the expression of osteogenic associated proteins. Interestingly, the seeding of coralline carbonate induced the lowest expression of osteogenic proteins indicating that complete hydrothermal conversion improves the osteoconductivity of the coralline material. Additionally the secretion profile showed high concentrations of a pro-inflammatory response across all seeded scaffolds in agreement with *in vitro* studies [423,436] however some authors have noted the pro-inflammatory environment supports tissue regeneration as it promotes downstream angiogenesis and initial bone regeneration [437-439]. This was evident in seeded nanoporous hydroxyapatite as it had the highest expression of pro-inflammatory cytokines while concurrently expressing high concentrations of vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF). These chemokines promote angiogenesis and subsequent vascularisation during bone fracture repair [440-442]. An *in vivo* animal model could potentially demonstrate the enhanced vascularisation of nanoporous HA implied by our findings.

The proteomic profile of seeded macroporous cHA expressed a large repertoire of cell adhesion and proliferation proteins suggesting that seeded ADSCs are exposed to a challenging microenvironment and need to proliferate across a macroporous scaffold where the pore interconnections can get as large as 500µm. Interestingly, the

secretion profile showed high concentrations of VEGF and G-CSF similarly to nanoporous cHA. This finding agrees with the Klenke and co-workers who demonstrated a relationship between pore size and vascularisation [443]. However, as previously discussed, nanoporous cHA seems to be mediating its proangiogenic activity through other mechanisms.

The investigation of seeded High Density cHA showed that the cells exhibited the expression of structural integrity proteins and extracellular matrix proteins. A subsequent literature review revealed a large number of these proteins played a role in bone morphogenesis. The presence of bone morphogenesis proteins clarifies a previous animal model which successfully utilised high density hydroxyapatite as an orthopaedic implant [444]. Additionally, the lack of osteoclast proteins identified indicates that high density cHA may have low bone resorbability as suggested by the previously mentioned study [444].

There is however, a concern for the low number of proteins identified from our proteomic analysis of the seeded coralline scaffolds. As previously mentioned, given the context that there are over 19000 genes within the human genome [429], we were only able to identify less than 500 proteins in total. We analysed the total ion count (TIC) of each respective sample in technical duplicates and found they all equally reached a peak TIC 10^6 with a similar elution profile (Supplementary Figure 5.1a-e). This suggests that insufficient amounts of total peptides were analysed during the MS analysis however this also shows that similar relative concentration of peptides were analysed.

The insufficient amount of peptides may have arisen from the low concentration of proteins obtained from our seeding of coralline scaffolds. This is due to the size of coralline scaffolds themselves as they are irregular in shape and constitute approximately 4mm x 5mm x 4mm in dimension. This allows only a small surface area of ADSCs to attach and proliferate in comparison to tissue culture flasks which generally constitute a surface area of 175cm^2 allowing more cells for growth and analysis.

5.4 Concluding remarks

This thesis has sought to characterise adipose derived stem cells coated onto coralline scaffolds as a potential bone tissue engineering construct. Bone related disorders such as osteoporosis and fractures are a huge burden on the Australian healthcare system [445]. The current gold standard of permanent metallic implants is at risk of imperfect bone union and in some cases implant infection and rejection[446]. Currently there are no effective alternatives that allow complete union of fractured bone and implant material. The work presented in this thesis has investigated ADSCs coated onto coralline scaffolds as a potential alternative to metallic implants due to the bio-resorbable and osteoconductive properties of coralline materials. The examination of the ADSCs seeded onto HA revealed a proteomic signature abundant in osteogenic proteins which suggested that coralline HA can induce a phenotypic change that is similar to that of bone cells.

This finding was further elaborated with a calcium ion dissolution test which detected small amounts of calcium to be released into the ADSC conditioned medium by coralline HA (Chapter 2). I assessed the indirect effect of HA on rADSCs and observed the presence of calcium deposition by rADSCs which indicates that the presence of calcium in the microenvironment is one of the factors influencing the osteoblast like phenotype I observed.

The proteomic examination of rADSC membrane subproteome revealed a number of proteins involved in ECM deposition, osteo-regulation and cellular adhesion however the presence of these proteins were only expressed after seeding ADSCs onto HA for 30 days (Chapter 3). I then investigated the effect of 5 different coralline materials, each with its own unique property on human ADSCs and found in agreement with my previous findings that coralline material can induce the expression of osteogenic proteins in hADSCs (Chapter 4).

A cytokine expression assay showed that macroporous and nanoporous cHA promotes

angiogenesis and inflammation in seeded HA ADSCs, a finding that has not been observed by other *in vitro* studies. Additionally, I showed for the first time that hydrothermally converted HA can induce a larger expression of osteogenic proteins than unconverted HA. Furthermore, I identified the presence of osteoclastic proteins in all our coralline cohorts suggesting that *in vivo* reports of bone resorption observed in HA implants may have occurred due to the coralline material itself.

Prior to this, no study had investigated the proteome of ADSCs seeded onto coralline hydroxyapatite and so my research detailed for the first time a change in the ADSC proteome that implies a phenotypic similarity to osteoblasts. The topics presented in this thesis have greatly expanded our understanding of the molecular effects of coralline materials on ADSCs that could potentially lay the foundation for future application in bone tissue engineering.

Appendix

Supplementary Table 4.1*:* Listed are some of the unique proteins identified from the 'Converted HA seeded ADSCs' cohort in comparison to the 'Unconverted coralline calcium carbonate seeded ADSCs' and 'ADSC' cohort. The proteins listed are sorted with their respective TrEMBL/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Protein Score	Coverage% (#unique peptides)	Role in Bone Morphogenesis
P02545	Lamin A/C	57	16.3	Lamin A/C, also known LMNA, is a component of the lamina and is required for the normal development of the skeletal system as it plays an important role in nuclear assembly during osteoblastogenesis and bone formation [352].
Q9Y490	Talin-1	60	3.7	Talin-1maintains the actin cytoskeleton adhesion by complexing with various integrins. During osteoclast activation,Talin-1 binds to macrophage colony stimulating factor (MCSF) and β integrin to mediate proper cytoskeleton rearrangement to ensure osteoclast viability [381].
Q00610	Clathrin heavy chain 1	194	6.9	Clathrin is a trimer of heavy chains that mediate endocytosis for membrane protein trafficking. It is involved in the uptake of ligand receptor complexes, membrane transporters and adhesion molecules [353].
P35232	Prohibitin	86	10.5	Prohibitin is a multi-functional membrane protein that has shown to increases cellular migration capacity and proliferation in mesenchymal stem cells [447].
P22314	Ubiquitin-like modifier-activating enzyme 1 (UBA1)	86	2.6	UBA1 activates the ubiquitination pathway which marks proteins for degradation and additionally facilitates cell cycle regulation and signal transduction for the WNT-CTNNB1/β pathway for osteoblast commitment in mesenchymal stem cells [448].
P39656	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase	69	12.9	Essential subunit of the N-oligosaccharyl transferase (OST) complex which catalyzes the transfer of a high mannose oligosaccharide and is found to be expressed in articular cartilage membranome [449].

	48 kDa subunit			
B7Z9L0	T-complex protein 1 subunit delta	47	16.8	T-complex protein 1 subunit molecular chaperone; assists the folding of proteins upon ATP hydrolysis. Known to play a role, *in vitro*, in the folding of actin and tubulin [450].
P50990	T-complex protein 1 subunit theta	1379	14.1	The theta subunit of the T complex chaperone protein which is involved in the folding of actin and tubulin after high metabolic activity such as ATP hydrolysis [450] A study investigating the potential biomarkers for osteonecrosis of the jaw found TCP1-θ to be downregulated in osteonecrotic patients [451].
P78371	T-complex protein 1 subunit beta (CCT2)	93	11.4	CCT2 is a component of the TCP1 ring complex and also performs the essential folding of actin and tubulin [452].
P17987	T-complex protein 1 subunit alpha (TCP1)	77	12.7	TCP1 is a component of the TCP1 ring complex and also performs the essential folding of actin and tubulin [452]
P04792	Heat-shock protein beta-1 (HSP β1)	92	11.2	HSP β1 is a small head shock protein that is involved in signal transduction, cytoprotection, chaperonin activity, cellular development and differentiation [453]. HSP27 has shown to be upregulated during osteogenesis as it mediates both cell differentiation and growth of osteoblasts [454].
Q92743	serine protease (HTRA1)	57	10.3	HTRA1 degradation of specific glycoproteins within the skeletal ECM has shown to play a central role in controlling mineral deposition by osteoblasts [321]. Importantly, the serine protease has been found to be a positive regulator of mesenchymal stem cell osteogenesis as the expression of HTRA1 increased in a time dependent manner [327]
P59998	Actin-related protein 2/3 complex subunit 4	66	17.8	Arp 2/3 complex protein activates actin filaments to maintain multiple forms of actin based structures that occur during the constant rearrangement of cytoskeletal framework of cells during cellular differentiation and migration[364] [365]. It's specific role in osteo differentiation however has not been elucidated.
I3L504	Eukaryotic translation initiation factor 5A-1	44	20.1	Eukaryotic translation initiation factor 5A-1 was identified as a critical protein involved in osteogenic differentiation of umbilical cord blood mesenchymal stem cells due to its 10 fold increase during induced bone morphogenesis[455].
P51149	Ras-related protein Rab-7a (RAB7A)	77	10.2	RAB7A facilitates endocytosis via endo-lysosomal trafficking. Additionally, RAB7A is abundantly expressed in ruffled border of osteoclasts where it facilitates bone resorption [456].

O43707	Alpha-actinin-4	121	8.4	ACTN4 anchors actin to a number of intracellular structures through the process of crosslinking ACTN 4 has been showed to play an important role in cellular adhesion and migration cell spreading and migration[457].
P81605	Dermcidin	72	17.3	Dermcidin has dual functions which include the 47 amino acid DCD-1 peptide that has shown to have antimicrobial activity[458] and the 30 amino acid PIF-CP peptide that mediates proteolytic activity [459]. The expression of PIF-CP occurs during mitochondrial stress which is initiated by calcium extrusion pathways at the plasma membrane [460].
P02771	Alpha-fetoprotein (AFP)	66	9.1	AFP is a plasma membrane protein binds to copper, nickel and fatty acids however its role in adult humans is not clear [461].
Q9NR31	GTP-binding protein SAR1a (SAR1A)	100	26.3	SAR1A is a GTP dependent transporter protein that translocates from the ER to the Golgi apparatus similarly to COPII mediated movement. In human osteoblasts,Sar1A is expressed. to promote the assembly of COPII vesicles during matrix deposition [462].
P49588	Alanine--tRNA ligase, cytoplasmic	49	11.2	Alanine-tRNA ligase is responsible for the attachment of alanine to tRNA during the process of protein translation. Additionally, Alanine-tRNA ligase has an editing domain which edits incorrectly charged tRNA. To date, there is no study which has linked Alanine-tRNA ligase to bone morphogenesis [463].
P23284	Peptidyl-prolyl cis-trans isomerase B (PPIB)	67	9.3	PPIB is involved in the cis-trans isomerization of proline rich residues of procollagen and collagen-1. Due to the reliance on PPIB to facilitate proper folding of collagen 1, mutations within the PPIB gene leads to severe osteogenesis imperfecta [464]
Q8WXE9	Stonin-2	53	4.3	Stonin 2 is an endocytic protein that interacts with the clathrin/AP-2 complex as an AP-2 dependent sorting adaptor protein which target synaptotagmins for endocytic internalisation[354]. Synaptotagmins enable calcium-induced exocytosis of essential ECM molecules in differentiating osteoblasts[355].
P13929	Beta enolase	53	4.3	Beta enolase is a glycolytic enzyme that innervates skeletal striated muscle during contraction and regenerates muscle tissue after fatigue [465] [466]. Beta enolase is a known marker for precursor myoblasts however its presence and role in bone morphogenesis Is unknown [467].
P01023	Alpha-2-macroglobulin	77	9.4	Alpha-2-macroglobulin is a protease which inhibits a number of proteolytic enzymes including metalloproteinases which are responsible for the degradation of ECM within skeletal tissue [468]. Additionally, alpha-2-macroglobulin is known to bind to a number of growth factors and cytokines including TFG-β and FGF [469].
P30048	Peroxiredoxin 3	60	12.6	PDX-3 is a redox regulator that protects the cell during high oxidative stress events such as cellular proliferation and differentiation. PDX-1 and PDX-5 have shown to be upregulated during osteogenesis however the role of PDX-3 during bone morphogenesis

				has not been revealed [470]..
Q9Y696	Chloride intracellular channel protein 4	118	20.2	Chloride intracellular channel proteins are expressed during hypercalcemic events which generally occur at bones and joints in the body [358]
Q9UI15	Transgelin-3	55	25.2	TAGLN3 is a microtubule associated protein has been described as a neuron specific protein in the developing neural tube [471]. A study investigating the effect of bone morphogenetic 7 (BMP-7) identified Transgelin-3 to be uniquely expressed in their treated cementoblast cells, eluding the idea transgelin 3 plays a role in actin binding during osteogenesis [472].
G3V3E6	ERO1-like protein alpha	103	11.7	ERO1-like protein alpha is an oxireductase enzyme which is heavily involved in disulfide bond formation of synthesised protein within the endoplasmic reticulum [473].
P21810	Biglycan, isoform CRA_a	71	10.4	Biglycan extracellular proteoglycan that positively regulates bone formation and osteoclast differentiation through its effects on osteoblasts and their precursors [382].
K7EJ44	Profilin 1	328	29.2	Profilin- 1 is a ubiquitously expressed G-actin protein that promotes actin polymerisation. It is essential for actin reorganization during osteoclast bone resorption [383]. Additionally, profilin is highly upregulated during BMSCs osteodifferentiation [303].
C1PHC2	CD44 molecule	69	8.6	CD44 is a multifunctional adhesion molecule that binds to hyaluronic acid. type 1 collagen, and fibronectin. It is found to be expressed on the plasma membrane of differentiating osteocytes as it is a core component of ECM regulation during bone morphogenesis[361].
P10276	Retinoic acid receptor alpha (RARA)	32	10.8	RARA acts as a ligand-dependent transcriptional activator to retinoic acid (RA), thus mediating RA's activity and effectiveness. Previous studies have established RA to play a significant role in osteoblast differentiation of ADSCs as it has shown to enhance proliferation and promote early BMP-2 induced osteoblastic differentiation [474].
Q96RR4	Calcium/calmodulin-dependent protein kinase II (CamKII)	53	5.1	CamKII is one of a family of calcium/calmodulin dependent protein kinases that play a central role in the transduction of calcium signals within the cell. During mechanical stimulation, CamKII is found to be expressed in normal osteo-articular tissue [359]`.
P06702	Protein S100-A9	100	36.8	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell migration and adhesion [408]. Additionally. chondrocytes express S100A9 and their upregulation is associated with IL-1, suggesting a role in cartilage repair during inflammatory induced tissue damage[475].
L8E9X3	Alternative protein CD19	50	12.9	CD19 is a heamapoetic marker generally absent in ADSCs and present in mostly B cell lymphocytes. The presence oMoesinf CD19 is indicative the ADSCs have undergone a phenotypic change as they no longer express CD marker characteristic of a MSC. The role of CD19 in ADSCs however has yet to be investigated.

P06756	integrin alpha-V isoform 2 precursor	64	2.9	The alpha-V integrins are receptors for vitronectin, fibronectin, osteopontin, osteomodulin and matrix mettaloproteinase-2 (MMP-2) to facilitate skeletal tissue repair [476]. IαV2 under goes post-transitional processing to create multiple isoforms that bind to extracellular matrix protein
Q92526	T-complex protein 1 subunit zeta-2 (TCP1-ζ2)	47	16.8	TCP1-ζ2 is a cytoplasmic molecular chaperone protein that assists in the folding of actin, tubulin, cyclin E and myosin. It interacts with up to 15% of the cellular actin and tubulin for effective protein folding[477]. Actin and tubulin act as an important perquisite for osteogenic MSC differentiation into osteocytes [478]
P05109	Protein S100-A8	59	26.9	Similarly to S100 A9, the S100A8 protein is a calcium binding protein that forms a complex with S100A9 to initiate NFκB and MAP kinase signaling which has shown top promote osteogenesis in MSCs [409] [410]. The role of S100A9 within the complex is to prevent S100A8 degradation as S100A is the active component of the complex [479].
P46934-4	Isoform 4 of E3 ubiquitin-protein ligase NEDD4	95	3.3	Isoform 4 of E3 ubiquitin-protein ligase NEDD4 is an isomer of Nedd4, an essential ligase for bone development as a study has shown that the absence of Nedd4 in pre osteoblasts results in impaired osteoblast proliferation and osteogenic differentiation. Furthermore, it regulates the Notch signaling pathway during intramembranous ossification [480]
O43707	Alpha-actinin-4 (ACTN4)	272	14.9	ACTN4 anchors actin to a number of intracellular structures through the process of crosslinking ACTN 4 has been showed to play an important role in cellular adhesion and migration cell spreading and migration[457].
Q9UKX2	Myosin-2	429	13.9	Myosin-2 is a motor protein that regulates cytoskeletal contraction through integrins and actins. During early osteodifferentiation of progenitor cells, myosin-2 is expressed to modulate critical ECM changes during osteogenesis [481].
B4DSD8	Alpha-1,4 glucan phosphorylase	144	5.7	Alpha-1,4 glucan phosphorylase releases alpha-D-glucose 1-phosphate by severing the alpha 1,4 link between pairs of glucose molecules at the end of long glucose polymers. This phosphorylases activity allows essential phosphorylation of glucose residues and thus plays a role in carbohydrate metabolism[482,483].
P37802	transgelin 2	74	31.4	The function pf transgelin 2 is unknown however, Sekigawa *et al* found that transgelin 2 was upregulated within the serum/plasma of osteoarthritic patients during infliximab treatment [484]. Additionally, Elsafadi *et al* showed that the gene responsible. for the transgelin family proteins are important for the for osteoblastic differentiation of human mesenchymal stem cells as it regulates cytoskeleton arrangement and distribution of actin filaments [485] .

Supplementary Table 4.2*:* Listed are some of the unique proteins identified from the 'Unconverted coralline calcium carbonate seeded ADSCs' cohort in comparison to the 'ADSCs' and 'Converted HA seeded ADSCs' cohort. The proteins listed are sorted with their respective TrEMBL/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
P08123	Collagen alpha-2(I) chain (ColA2)	63	2.6	Collagen alpha 2 combines with pro alpha 1 chain to form type 1 procollagen which becomes collagen fibers before becoming mineralised by calcium hydroxyapatite [360].
O00445	Synaptotagmin-5	73	3.6	Synaptotagmin-5 mediates calcium-dependent binding at synaptic vesicles for calcium-induced exocytosis of essential ECM molecules in differentiating osteoblasts [311].
P26038	Moesin	60	9.7	Moesin is a member of the ERM protein family which acts as a cross linker between the plasma membrane and the cytoskeleton of a cell which allows cell to cell signaling. Moesin and CD44 are known to form a protein complex within osteoclasts. This CD44-moesin-actin filament system allows the osteoclasts to recognize the bone surface and initiate bone resorption [361] [362].
P17844	Probable ATP-dependent RNA helicase DDX5	95	9.3	DDX5 is a multifunctional DEAD box protein that regulates gene expression by affecting RNA processing and transcriptional initiation. DDX5 enhances Runx3 transcriptional activity and thus increases osteoblast differentiation [376].
Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	59	6.6	Procollagen-lysine,2-oxoglutarate 5-dioxygenase (PLOD-3) post transitionally modifies collagen through hydroxylation of lysine residues leading to the binding and cross-linking of fibrils to collagen fibers. This provides tensile strength to collagen fibers within the organic matrix of bone[378].
Q14213	Interleukin-27 subunit beta	45	3	Interleukin-27 is cytokine that is structurally related to IL-2 and IL-23. Molecular entities such as p68 are essential for formation of functional IL-27[389]. Studies

				have shown that IL-27 plays a major role in the regulation of osteoclastogenesis as it affects both RANKL dependent and independent pathway [390]s.
Q09666	Neuroblast differentiation-associated protein AHNAK (NdapA)	72	3.3	NdapA is a chaperone regulatory protein whose molecular function is unknown. However, studies have implicated the protein to play a role in osteoblasts as it is upregulated during endothelial-1 treatment of calvarial cells and osteoblast Saos-2 cell lines [486] [487].
P30041	Peroxiredoxin-6	70	45.4	PDX6 is an enzyme that has both glutathione peroxidase and lysosomal type phospholipase A2 activities, and it also possess an antioxidant activity. The peroxidase family is heavily involved in the regulation of ROS activity within the body. The increase in peroxidase expression suggests it acts as a protective measure against ROS mediated tissue damage during osteoarthritis.[488]
Q15019	Septin 2	36	11.4	Septin-2 is similar to Nedd 5 and is essential for the formation of a primary cilium, which is a protruding filament onto the extracellular space from the cell surface [489]. Studies have found septin-2 to be upregulated in osteoblasts during osteogenesis as it promotes the formation of the primary cilium which is essential for growth factors to stimulate bone growth [490].
P11717	Cation-independent mannose-6-phosphate receptor	59	2	Also known as insulin like growth-factor 2 receptor, this protein regulates ECM deposition in osteoblast like cells by conversely inhibiting insulin growth-factor 1 [363].
V5YQU3	FGF-BICC1 fusion kinase protein	72	6.1	A fusion protein of the fibroblast growth factor (FGF) family of proteins which is responsible for cellular migration and differentiation [491]. The role of FGF-BICC1 fusion kinase protein in osteodifferentiation is yet to be elucidated.

Supplementary Table 4.3: Listed are some of the unique proteins identified from the 'Macroporous seeded ADSCs' cohort in comparison to the 'Nanoporous ADSCs' and 'ADSC' cohort. The proteins listed are sorted with their respective TrEMBL/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
P59998	Actin-related protein 2/3 complex subunit 4 (Arp 2/3 complex)	46	14.3	Arp 2/3 complex protein activates actin filaments to maintain multiple forms of actin based structures that occur during the constant rearrangement of cytoskeletal framework of cells during cellular differentiation and migration[364] [365]. Its specific role in osteo differentiation however has not been elucidated.
P06703	Protein S100-A6 (Calcyclin)	63	8.9	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell adhesion and differentiation [369]. Protein S100-A6, also known as calcyclin is responsible for sensing extracellular calcium and other cations such as aluminum and magnesium to regulate the osteoblast function [370].
P01023	Alpha-2-macroglobulin	77	9.4	Alpha-2-macroglobulin is a protease which inhibits a number of proteolytic enzymes including metalloproteinases which are responsible for the degradation of ECM within skeletal tissue [468]. Additionally, alpha-2-macroglobulin is known to bind to a number of growth factors and cytokines including TFG-β and FGF [469].
B4DHZ6	Transferrin, isoform CRA_c	65	6.5	Transferrin is a glycoprotein which can readily bind iron and in the absence of iron is called apoprotein which interacts with insulin like growth factors (IGF) to modulate IGF functions such as osteogenesis and chondrogenesis[492] [493].
Q16658	Fascin homolog 1, actin-	107	18.5	Fascin is an actin binding protein which co-localises actin filaments to the

	bundling protein			periphery of the cell to meditate cell adhesion [366]
P60981	Destrin	75	20	Destrin regulates disassembly and assembly of actin filaments which are essential for cellular membrane ruffling and extension, a process common in osteoblast differentiation into osteocytes during bone development[322].
P40227	T-complex protein 1 subunit zeta	68	17.1	A molecular chaperone that assists in the folding of actin and tubulin *in vitro*[367], The proper folding of actin and tubulin is essential for many biological processes however its role in osteogenesis has not been elucidated.
P35749	Myosin-11	429	17.9	Myosin-11 is a hexameric contractile protein that converts the energy generated from ATP hydrolysis into mechanical contraction within smooth muscle tissue. Additionally, myosin 11 participates in the stabilisation of osteoclast lamellipodium which allows the osteoclasts to resorb substrate within the lacunae of bone [388].
P11047	Laminin subunit gamma-1 (Lγ-1)	100	5.9	Lγ-1 binds to skeletal ECM components with high affinity to mediate attachment and organisation of neighboring cells. Additionally, Lγ-1, Lα-1 and Lβ-1 has been found to be differentially expressed during osteogenesis [368]
I3L504	Eukaryotic translation initiation factor 5A-1	89	15.1	Eukaryotic translation initiation factor 5A-1 was identified as a critical protein involved in osteogenic differentiation of umbilical cord blood mesenchymal stem cells due to its 10 fold increase during induced bone morphogenesis[455].

Supplementary Table 4.4*:* Listed are some of the unique proteins identified from the 'Nanoporous seeded ADSCs' cohort in comparison to the 'Macroporous seeded ADSCs' and 'ADSC' cohort. The proteins listed are sorted with their respective TrEMBL/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
Q7RTY7	Ovochymase-1	55	1.4	Ovochymase-1 is a chymotrypsin like protease which has been characterized to play a role in oocyte development and was found to be secreted onto the cell surface [494].
Q15149	Plectin	57	3.8	Plectin is a cytoskeletal cross linking protein which maintains mechanical integrity of many skeletal tissues, including muscle and bone. Additionally, the inhibition plectin has shown to bypass cellular senescence in rat fibroblasts [373].
P26447	Protein S100-A4	63	8.9	S100A4, also known as calvasculin, a calcium binding protein that interacts with microtubilin, actin and myosin to regulate cytoskeletal dynamics and elements such as cellular proliferation[371]. Interestingly, S100A4 has known to upregulate MMP-13 to stimulate chondrocyte differentiation [372].
P06703	Protein S100-A6	65	8.9	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell adhesion and differentiation [369]. Protein S100-A6, also known as calcyclin is responsible for sensing extracellular calcium and other cations such as aluminum and magnesium to regulate the osteoblast function [370].
P06756	integrin alpha-V isoform 2 precursor (IαV2)	68	4.6	The alpha-V integrins are receptors for vitronectin, fibronectin, osteopontin, osteomodulin and matrix mettaloproteinase-2 (MMP-2) to facilitate skeletal tissue repair [476]. IαV2 under goes post-transitional processing to create multiple isoforms that bind to extracellular matrix proteins [495]

G3V3E6	ERO1-like protein alpha	113	9.2	ERO1-like protein alpha is an oxireductase enzyme which is heavily involved in disulfide bond formation of synthesised protein within the endoplasmic reticulum [473].
Q02818	Nucleobindin-1	91	8.2	Nucleobindin 1 is a multifunctional calcium binding protein that was identified in the ECM of bone matrix and was later termed calnuc [374]. Within the bone matrix, calnuc binds and deposits calcium to form hard tissue. Additionally, calnuc binds hydroxyapatite to import calcium inside osteoblasts and osteocytes [375].
B3V096	BetaCstF-64 variant 2	60	9.9	BetaCstF-64 is involved in tissue specific polyadenylation which contributes to proteomic diversity to allow specialised functions of particular proteins [496]. Its role in osteogenesis is not known.
P20700	Lamin B1	48	5.9	Lamin B1 is a multifunctional nuclear intermediate filament that supports the structural integrity of the nucleus, and play a key role in stem cell niche function such as cell proliferation and differentiation of specific lineages, including bone formation [377]. A high ratio of lamin B is correlative to the stiffness of a cell and thus bone tissue is highly abundant in lamin A and lamin B [225].
P52907	F-actin-capping protein subunit alpha-2 (CapZA2)	86	9.4	CapZA2 is a F-actin capping protein CapZA2 binds the barbed end of actin filaments and prevents addition or loss of actin monomers to filaments in c calcium dependent manner which is why it is commonly expressed in skeletal muscle [497].
P27824	calnexin, partial	86	16.6	Calnexin is a calcium binding protein commonly expressed within the microvesicles of osteoblast like cells, called Saos-2 cells. It's thought that calnexin is an important component in the mineralisation process of HA within the lumen of differentiating Saos-2 cells [324].
P13929	Beta enolase	240	33.9	Beta enolase is a glycolytic enzyme that innervates skeletal striated muscle during contraction and regenerates muscle tissue after fatigue [465] [466]. Beta enolase is a known marker for precursor myoblasts however its presence and role in bone morphogenesis Is unknown [467].
P30048	Peroxiredoxin 3	70	45.4	PDX-3 is a redox regulator that protects the cell during high oxidative stress events such as cellular proliferation and differentiation. PDX-1 and PDX-5 have shown to be upregulated during osteogenesis however the role of PDX-3 during bone morphogenesis has not been revealed [470]..

Supplementary Table 4.5: Listed are some of the unique proteins identified from the 'High Density seeded ADSCs' cohort in comparison to the 'Unconverted coralline calcium carbonate seeded ADSCs' and 'ADSCs' cohort. The proteins listed are sorted with their respective TrEMBL/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
O43776	Asparagine--tRNA ligase, cytoplasmic (NARS)			NARS is responsible for the attachment of asparagine to tRNA during the process of protein translation and is heavily involved in N-linked glycosylation of proteins. Furthermore, Park *et al* was able to show NARS to be crucial for the survival of FGF-2 induced osteoblasts [41].
P17844	Probable ATP-dependent RNA helicase DDX5	95	9.3	DDX5 is a multifunctional DEAD box protein that regulates gene expression by affecting RNA processing and transcriptional initiation. DDX5 enhances Runx3 transcriptional activity and thus increases osteoblast differentiation [376].
Q61576	FK506 binding protein 10, 65 kDa, isoform CRA_b	102	8	FKBP is a protein that has prolyl isomerase activity that coordinates cytoskeletal organisation through the transport of early endosomes between microfilaments. Mutations within the FKBP gene leads to development of osteogenesis imperfecta as the proper folding of the collagen triple helix does not occur for procollagen 1 secretion during skeletal development [379].
Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	59	6.6	Procollagen-lysine,2-oxoglutarate 5-dioxygenase (PLOD-3) post transitionally modifies collagen through hydroxylation of lysine residues leading to the binding and cross-linking of fibrils to collagen fibers. This provides tensile strength to collagen fibers within the organic matrix of bone[378].
P30050	Ribosomal protein L12 (RPL-12)	62	13.3	RPL-12 acts a component of the 60S and is essential for optimal ribosome function in protein biosynthesis. RPL-12 is acetylated to produce RPL-7 to increase cell proliferation [498].
P52597	Heterogeneous nuclear	208	12.8	Together with hnRNPC and hnRNR, hnRNPF is a component of the hnRNP

	ribonucleoprotein F (hnRNPF)			complex for pre-mRNA processing. Interestingly, hnRNPF was identified as a novel inhibitor of fibroblast growth factor receptor 2 (FGFR2). FGF and FGFR2 plays a major role as a positive regulator of bone formation [499].
P53396	ATP-citrate synthase	92	10.1	ATP-citrate synthase synthesizes cytosolic acetyl-CoA and plays a role in *de novo* lipid synthesis. This provides an alternative source of energy production from citrate metabolism which leads to the development of stem cells to specialized functional cells [500].
P38117	Electron transfer flavoprotein subunit beta	81	13.6	Electron transfer protein (ETF) is a mobile electron carrier that transfers electrons between several flavin adenine dinucleotide (FAD)-containing dehydrogenases that are present throughout the mitochondrial matrix. ETF consists of two subunits called the alpha and beta subunit which cohesively act together as a FAD containing dehydrogenase [501].
P10301	Ras-related protein R-Ras	150	12.8	R-Ras promotes cellular adhesion through the regulation of the actin cytoskeleton [502]. Additionally, R-Ras has shown to interact with integrin beta-1 to mediate its cellular adhesion and migration capacity [503]. Additionally, R-Ras signaling has shown to induce osteogenesis in BMSCs [504].
P0DMV8	Heat shock protein 70 (HSP70)	139	13.4	HSP70 is a chaperone protein that mediates protein folding during cellular stress events. Interestingly, Yao et al showed HSP70 binds to matrix Gla protein to promote BMP signaling, allowing calcification to occur in calcifying vascular cells *in vitro* [505].
Q02818	Nucleobindin-1	84	17.7	Nucleobindin 1 is a multifunctional calcium binding protein that was identified in the ECM of bone matrix and was later termed calnuc [374]. Within the bone matrix, calnuc binds and deposits calcium to form hard tissue. Additionally, calnuc binds hydroxyapatite to import calcium inside osteoblasts and osteocytes [375].
P26447	Protein S100-A4	63	8.9	S100A4, also known as calvasculin, a calcium binding protein that interacts with microtubilin, actin and myosin to regulate cytoskeletal dynamics and elements such as cellular proliferation[371]. Interestingly, S100A4 has known to upregulate MMP-13 to stimulate chondrocyte differentiation [372].
P20700	Lamin B1	48	5.9	Lamin B1 is a multifunctional nuclear intermediate filament that supports the structural integrity of the nucleus, and play a key role in stem cell niche function such as cell proliferation and differentiation of specific lineages, including bone

				formation [377]. A high ratio of lamin B is correlative to the stiffness of a cell and thus bone tissue is highly abundant in lamin A and lamin B [225]
P09525	Annexin A4	133	20.4	Annexins are well-characterized in bone as components of mineralizing matrix vesicles[300]. Annexin A4 is a calcium dependent phospholipid membrane binding protein modulates NF-κβ signalling by directly interacting with the p50 heterodimeric p50/65 NF-κβ complex which participates in the RANKL pathway of osteoclast regulation [301] [302].
O60763	General vesicular transport factor p115 (GVTF-p115)	63	5.8	GVTF-p115 is a membrane transport factor that translocates from the cytosol to the Golgi membrane during interphase to mediate the compartmentalization of secretory proteins [506]. It's participation in bone morphogenesis however is yet to be elucidated.
P52209	HUMAN 6-phosphogluconate dehydrogenase, decarboxylating (PGD)	202	11.4	PGD is an oxireductase enzyme that is involved in the nucleotide synthesis through the generation of ribulose 5-phosphate and acts in the pentose phosphate pathway for the production of cellular NADP [507].
P30041	Peroxiredoxin-6	183	21.7	PDX6 is an enzyme that has both glutathione peroxidase and lysosomal type phospholipase A2 activities, and it also possess an antioxidant activity. The peroxidase family is heavily involved in the regulation of ROS activity within the body. The increase in peroxidase expression suggests it acts as a protective measure against ROS mediated tissue damage during osteoarthritis.[488]
B3KRY5	cDNA FLJ35087 fis, clone PLACE6005546, highly similar to Polymerase I and transcript release factor	84	7	Cavin interacts with caveolin-1 in the formation and organisation of the caveolae within the osteoblast membrane where the protein complex regulates the function of a number of signalling molecules and receptors. [508] [509] [510].
O75369	FLNB_HUMAN Filamin-B	191	6.1	Filamin B cross-links actin which enables intracellular communication between the cytoskeleton and cell membrane during skeletal development. Various studies have shown that a mutation within the filamin B gene can lead to an impairment of bone formation [380]
B4DSU6	Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC)	80	19.7	HNRNPC binds single strand polynucleotides and contributes to pre-MRNA processing and stabilization during nuclear translation and export [511]. HNRNPC has been shown to modulate the steroid hormone 1,25-dihydroxyvitamin D(3) (calcitriol) through its interaction with vitamin D response ligands [512]. This allows HNRNPC to play a critical role in the regulation of bone

				homeostasis and act as a modulator of bone morphogenesis [513].
Q9UHV8	beta-galactoside-binding lectin, partial	123	35.8	During the matrix maturation stage of osteoblastic development, beta-galactoside-binding lectin was found to be upregulated in a study investigating the protein profiling of osteo-differentiated NSQ50 cell lines [514]
P2940	Transketolase	65	6.1	Transketolase activity requires divalent metal ions and thiamin diphosphate. It controls the non-oxidative part of the differentiation of mesenchymal stem cells into osteoblasts [515]
Q99714	3-hydroxyacyl-CoA dehydrogenase	80	14.4	3-hydroxyacyl-CoA dehydrogenase is an oxireductase enzyme that catalyzes the third step in the beta-oxidation of fatty acid metabolic processes [516]
B4DLR8	NAD(P)H dehydrogenase [quinone] 1 (NQO1)	75	11.3	NQO1 is a dehydrogenase enzyme that reduces quinones into hydroquinones and is also involved in production of prothrombin, vitamin K1,K2 and K3 [517]. Additionally, NQO1 carboxylates specific glutamic acid residues within Gla-related proteins to mediate their participation in bone metabolism and wound healing [518].
P04075	Fructose-bisphosphate aldolase A (aldose)	103	16.3	Aldose plays an important role in glycolysis and gluconeogenesis as it drives the forward and reverse reactions of both these metabolic pathways.. During bone resorption, aldose has been shown to activate vacuolar H+-ATPase to allow essential proton secretion from the osteoclast plasma membrane [519].
P35564	calnexin, partial	86	13.6	Calnexin is a calcium binding protein commonly expressed within the microvesicles of osteoblast like cells, called Saos-2 cells. It's thought that calnexin is an important component in the mineralisation process of HA within the lumen of differentiating Saos-2 cells [324].
D3ZD97	DEAH (Asp-Glu-Ala-His) box polypeptide 15	93	4.6	DEAH box proteins are a class of enzymes which heavily regulate post transcriptional modifications of RNA. Due to a large array of possible DEAH proteins within the human genome[520]. it is thought the expression of one is indicative of a specialized role. The function of DEAH (Asp-Glu-Ala-His) box polypeptide 15, isoform however, is unknown.
F8WAR4	MICOS complex subunit	60	14.5	MICOS-MIC19 is a component of the MICOS complex which constitutes the inner boundary membrane and the cristae junctions of mitochondria and acts as the scaffolding for the formation of contact sites for mitochondrial function [521]. Recently, MICOS was found to be down regulated during myocardiogenesis of embryonic stem cells however, the molecular mechanism

				through which this occurred was not explained [522]. Additionally, no studies have identified MICOS complex subunit as an component for bone morphogenesis.
Q92882	Osteoclast-stimulating factor 1 (OsF-1)	43	4.7	OsF-1is an intracellular protein that induces osteoclast formation and the resorption of inorganic hydroxyapatite. Additionally, OsF-1is produced by osteoclasts to mediate its bone homeostasis effects via signaling complexes with its diverse biological partners [523].
Q15046	Lysyl-tRNA synthetase	49	11.1	Lysyl-tRNA synthetase is an aminoacyl-tRNA synthetases that is localised within the multi synthetase complex (MSC) where it acts as a translation apparatus within the ribosome [524]

Supplementary Table 4.6*:* Listed are some of the proteins shared between the 'ADSC converted HAP seeded' cohort, 'ADSC unconverted HAP seeded'cohort and 'ADSC' cohort. The proteins listed are sorted with their respective trembl/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
P35579	Myosin-9	549	17.1	Myosin-9 is a non-muscle myosin heavy chain (myosin II) and is normally expressed in the cell cortex and stress fibers of non-migrating cells in vertebrates. Its role in osteogenesis however is unknown[525].
P68104	Elongation factor 1-alpha 1 (eEF1A)	453	32.5	The role of eEF1A in cytoskeleton organization may promote bone morphogenesis as eEF1A1 is over expressed in osteoblasts which induces proliferation and differentiation during skeletal repair [526] [527].
P14625	Endoplasmin	309	259	Endoplasmin, also known as GRP94 is found to be abundantly expressed in Saos-2 cells during calcium deposition. Along with other calcium binding chaperone proteins translocate towards the extracellular matrix as a complex. This mechanism continues to be important in maintaining intracellular and extracellular calcium homeostasis [324,528] .
P21333	Filamin-A (FLNA)	687	14.8	FLNA is an actin binding and cross linking protein which regulates the cytoskeletal network to regulate signal transduction during cell migration [386]. Filamin A is required for osteoclastogenesis as FLNA null progenitor cells cannot undergo osteoclastogenesis *in vivo* and *in vitro*. Furthermore, FLNA null animal models show skeletal abnormalities and skeletal dysplasia [387].
P08670	Vimentin	667	48.9	Vimentin is an important component of the mesenchymal cell cytoskeleton as it supports the organelles within the cytosol [529]l. Although it is present within all cells of the mesenchyme such as bone, the role it plays during bone

				morphogenesis is unknown.
P07355	Annexin A2	448	32.4	Annexin A2 plays a crucial role in bone morphogenesis by regulating the migration and adhesion of heamapoetic stem cells to osteoblasts and endothelial cells [530]. Additionally, Annexin A2 deficient mice showed impaired osteoblast differentiation [300].
P08133	Annexin A6	64	15.3	Annexin A6 is a cytoplasmic protein that translocates and binds to the membrane in the presence of extracellular calcium. Within the membrane, annexin A6 is involved in fibrinolysis and osteo-articular formation through the modulation of the NF-kB signaling pathway in the presence of TNFα and IL-1β [531] .
Q9NZ23	Drug-sensitive protein 1	207	57.4	Thought to act as an oxidoreductase enzyme involved in the reduction process via NAD or NADPH acceptor of aldehyde donors [532]. The function of Drug-sensitive protein 1 is yet to be determined.
Q14315	Filamin-C	475	12.8	Filamin-C is a large cytoplasmic protein that cross links cortical actin filaments of striated muscle tissue. Additionally, filamin-C interacts with a number of integrin receptors (Calpain-3) to trans extra cellular signals; however its role within skeletal tissue is unknown [533].
P13639	Elongation factor 2 (EF-2)	100	12	EF-2 is allows the GTP dependent translocation of proteins from the ribosome and thus is important for protein synthesis [534]. Kitching *et al* identified EF-2 to be upregulated during the matrix deposition and early osteogenic differentiation phases of MC3T3-E1 cells [535] .
D6RBL5	Annexin (AnxA5)	49	6.9	Annexin A5 is a calcium binding protein which acts as a channel to allow an influx of calcium ions during hydroxyapatite formation. AnxA5 is upregulated by osteoblasts during osteogenesis to allow endochondral ossification through the aforementioned mechanism [536].
P04083	Annexin A1	286	18.5	Annexin A1 binds to the cellular membrane in calcium dependent manner in a similar fashion as the other annexins from the same superfamily of proteins [537]. The molecular function of annexin 1 ranges from regulating the cytoskeletal rearrangement of MSCs and osteoblasts during osteogenesis and to inhibit spontaneous differentiation of MSC populations[538].
Q99623	Prohibitin-2	195	38.1	PHB2 modulates estrogen receptor alpha to regulate RunX2 transcription which accelerates and initiates osteoblastogenesis[539].

Q5JP53	Tubulin beta chain	953	32.2	Consists of the two chains of microtubules that bind GTP on an exchangeable site on the beta chain and non-exchangeable on the alpha chain [540]. Its role in bone morphogenesis is yet to be highlighted.
P38646	Stress-70 protein, mitochondria (Hspa9a)l	199	18.1	A chaperone protein which controls cellular proliferation and recently was identified as an osteocyte selective protein which aids in the cytoskeleton protein folding [541].
P60842	Eukaryotic initiation factor 4A-I (eIF4A).	92	16	Binds 40S RNA for eukaryotic translation of proteins. A study found eIF4A to be upregulated in zinc coated ceramics seeded [542]
H7BZ94	Protein disulfide-isomerase	129	15.5	Protein disulfide isomerase is an essential protein for the biosynthesis of procollagen, an important component of cortical bone. The enzyme protein disulfide isomerase associates the inter and intra of the procollagen chains [543].
5030431	Vimentin	667	48.9	Vimentin is an important component of the mesenchymal cell cytoskeleton as it supports the organelles within the cytosol [529]l. Although it is present within all cells of the mesenchyme such as bone, the role it plays during bone morphogenesis is unknown.
P02452	Collagen alpha-1(I) chain	43	2.7	Collagen alpha 2 combines with pro alpha 1 chain to form collagenase and type 1 procollagen which becomes mineralised by calcium hydroxyapatite to form bone collagen fibers [360].
O00469	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	132	8.6	Procollagen-lysine,2-oxoglutarate 5-dioxygenase (PLOD-3) post transitionally modifies collagen through hydroxylation of lysine residues leading to the binding and cross-linking of fibrils to collagen fibers. This provides tensile strength to collagen fibers within the organic matrix of bone[378].
P26641	Elongation factor 1-gamma (Ef1-γ)	45	6.4	Ef1-γ is involved in the biosynthesis of proteins during chondrogenesis however its role in osteogenesis has yet to be elucidated[544].
B7Z992	FLJ53698, highly similar to Gelsolin	124	10.7	Isoforms of gelsolin are an actin regulating protein for osteoclast precursor cells [241]. During osteoclast maturation, osteopontin regulates the interaction of gelsolin to form cytoskeletal rings important for osteoclast formation during bone remodeling [298].
P19367	Hexokinase-1	71	7	Hexokinase-1 phosphorylates glucose to synthesise glucose-6-phosphate for the production NADPH during high energy metabolic events such as cellular differentiation in pluripotent stem cells [545] .

P60709	Actin, cytoplasmic 1 (AC-1)	1991	56.5	AC-1 is ubiquitously expressed in all eukaryotic cells as it is an important conserved protein which aids in cell motility. Interestingly, AC-1 has found to be upregulated during dexamethasone induced osteogenesis in MC3T3-E cells [546].
P49368	T-complex protein 1 subunit gamma (TCP-3)	107	14.3	A chaperone protein involved in the folding of actin and tubulin after high metabolic activity such as ATP hydrolysis [450] A study investigating the negative effects of an anti-osteogenesis agent, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) on mesenchymal stem cells revealed that TCP-3 was severely downregulated after TCDD treatment, instigating TCP-3 might play a role in bone morphogenesis [547] .
P06733	Alpha-enolase	66	14.5	Enolase 1 is a multifunctional protein that is induced by pro-inflammatory stimuli and expressed during cellular differentiation [548]. Additionally, it has found to be overexpressed in rheumatoid arthritis fibroblasts -like synoviocytes and play a role in glycolysis [549].
P15121	Aldose reductase	60	12.2	An important enzyme for the NADPH dependent synthesis of fructose.. The production of fructose is an alternative energy production pathway during high metabolic events and a recent study has shown aldose reductase expression inhibits adipogenesis within 3T3-L1 cell to ensure hADSCs homogeneity [550].
F5H5D3	Tubulin alpha-1C chain	1329	36.2	The alpha chain component of the tubulin protein, which is responsible for the cytoskeleton regulation of synovial tissue cells surrounding bone. Its importance in osteo-maintenance has been highlighted by studies which found Tubulin alpha-1C chain to be down regulated in osteoarthritic induced tissue[551] [552].
E9PK25	Cofilin-1	106	16.8	Cofilin 1 aggregates with actin to regulate cytoskeleton dynamics which play a major role in osteoblasts during cytoskeleton stress events such as osteodifferentiation [546] [553].
P37802	Transgelin 2,	74	31.4	The function pf transgelin 2 is unknown however, Sekigawa *et al* found that transgelin 2 was upregulated within the serum/plasma of osteoarthritic patients during infliximab treatment [484]. Additionally, Elsafadi *et al* showed that the gene responsible. for the transgelin family proteins are important for the for osteoblastic differentiation of human mesenchymal stem cells as it regulates cytoskeleton arrangement and distribution of actin filaments [485] .

P02751	Fibronectin	694	11.5	Essential for osteoblast mineralisation, fibronectin and all its associated isoforms are involved in the compaction osteoblast compaction through cell mediated fibrillogenesis in the ECM of skeletal bone. Additionally, fibronectin participates in the modulation of type I collagen secretion by osteoblasts during osteogenesis. Additionally, fibronectin has shown promote osteoconductive properties in conjunction with vimentin on a human osteoblast cell line[240].
B4DHZ6	Transferrin, isoform CRA_c	65	6.5	Transferrin is a glycoprotein which can readily bind iron and in the absence of iron is called apoprotein which interacts with insulin like growth factors (IGF) to modulate IGF functions such as osteogenesis and chondrogenesis[492] [493].
Q562R1	Beta-actin-like protein 2	514	25.5	Beta actin like protein 2 is a cytoskeletal regulatory protein, similarly to tubulin beta chain [554]. Their role together is to promote cellular structural integrity however both proteins have no identified role in bone morphogenesis.
C9J5W0	Translocon-associated protein subunit-alpha (TRAP-α)	109	5.6	TRAP-α is part of a complex which binds calcium to the endoplasmic reticulum membrane to regulate the retention time of ER resident proteins. Due to its ER location, it may be involved in the translocation process of protein folding and recycling [555]
119606362	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12), isoform CRA_e	64	2.9	Beta 1 integrin binds to the major ECM component of bone, collagen 1 which is why beta 1 integrin is a crucial regulator of osteogenesis and mineralisation *in vitro*[556]. Additionally, Shekaran et al showed beta 1 integrin expression to be essential in early mesenchymal condensations for skeletal ossification as beta 1 integrin ECM interactions in pre osteoblast lineage cells regulate endochondral incisor eruption and perinatal bone formation [557].
Q9BX84	Transient receptor potential cation channel subfamily M member 6 (TRPV6)	43	3.5	(TRPV6) is an apical membrane calcium channel protein which is essential for vitamin D regulated calcium absorption [558]. Vitamin D regulated absorption of calcium plays a vital role for the homeostasis of skeletal tissue cells as knock out studies of the TRPV6 has shown a reduced bone thickness in mice models [559].
P60981	Destrin	73	16.4	Destrin regulates disassembly and assembly of actin filaments which are essential for cellular membrane ruffling and extension, a process common in osteoblast differentiation into osteocytes during bone development[322].
P12235	ADP/ATP translocase 1	77	21.1	ADP/ATP translocase 1 is a transporter protein that exchanges cytosolic ADP and ATP with the inner membrane of the mitochondrial wall [560]. This exchange allows efficient metabolic processes to continue such as bone

				formation as Lehenkari *et al* pointed out that inhibition of ADP/ATP translocase 1 through the use of bisphosphonates can lead to osteonecrosis [561].
A0A0B4J269	Tubulin beta-3	1389	14.1	Components of tubulin are a major constitute of cellular microtubules. The function of Tubulin beta 3 chain is unknown.
P27348	14-3-3 protein theta	111	22.9	14-3-3 protein theta is a regulatory protein which binds an array of signaling molecules such as phosphatases, transmembrane receptors and kinases [562] and has found to be upregulated during osteogenesis of hBMDMSCs [563].
P31947	14-3-3 protein sigma	85	16.9	An isoform of the 14-3-3 protein family of proteins, 14-3-3 protein sigma is unique in a sense as it has found to be expressed in osteo-articular cells during induced compression highlighting a role in the maintenance of articular osteoblasts during chronic compression [564]
O96019	Actin-like protein 6A (ACTL6A)	831	33.5	ACTL6A alters the DNA nucleosome topology to suppress selective genes and is also involved in transcriptional activity [356]. Additionally, ACTL6A was found be expressed by somatic adult precursor cells to control nucleosome associated differentiation [357].

Supplementary Table 4.7*:* Listed are some of the unique proteins identified from the 'ADSCs' cohort in comparison to the 'Converted HAP seeded ADSCs' and 'Unconverted HAP seeded ADSCs' cohort. The proteins listed are sorted with their respective trembl/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Protein Score	Coverage% (#unique peptides)	Role in Bone Morphogenesis
384381544	beta-tubulin 4Q isoform 1, partial	52	7.3	Beta-tubulin is a major constituent of the cellular cytoskeleton. Specifically, beta-tublin 4Q id highly expressed in the cell bodies of the central nervous system [565].
Q96RT7	Gamma-tubulin complex component 6 (GTCP6)	51	2.1	GTCP6 is one of the major components of the gamma tubulin complex which is responsible for the nucleation of the centrioles in the cell.GTCP6 has not definitive role during osteogenesis [566].
P11717	Cation-independent mannose-6-phosphate receptor	59	2	Also known as insulin growth-factor 2 receptor, this protein regulates ECM deposition in osteoblast like cells by conversely inhibiting insulin growth-factor 1 [363].
O14983	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1, partial	50	5.2	Regulates cytosolic calcium through ATPase reuptake of calcium into the sarcoplasmic reticulum for striated muscle activation. This consequently sequesters calcium for improved muscle function [567].
4261577	CD8 beta chain	50	30	An isoform of the transmembrane glycoprotein CD8 molecule, it is commonly expressed for cytotoxic T cell regulation. The presence of CD8 beta chain on MSCs is hypothesized to play a role on the immunosuppressive role of MSCs on T-lymphocyte proliferation [568].
119617396	low density lipoprotein-related protein 1 (alpha-2-	117	3	Low density lipoprotein related protein 1 (LRP1) regulates the composition of the plasma membrane to express a number of surface proteins such as beta integrin 1

	macroglobulin receptor), isoform			which mitigates cellular adhesion and migration [569].
P49755	Transmembrane emp24 domain-containing protein 10 (TMED10)	30	5.3	TMED10 is a vesicular trafficking protein that functions in the early secretory pathway at the lumenal side of the plasma membrane where it is involved in the vesicle coat formation. TMED10 does not play a role in osteogenesis however a study identified TMED10 to be overexpressed in individuals with prominent long bones [570].
A8MT65	Zinc finger-like protein 891 (ZFLP-891)	52	2.9	Zinc finger like proteins are transcriptional regulators of individual proteins. The role of ZFLP-891 however is unknown as its characterized role has not been determined. It's similarity to C2H2 zinc finger like protein however may elucidate a role in inhibiting osteoclast genesis [571].

Supplementary Table 4.8: Listed are some of the unique proteins identified from the 'Converted HAP seeded ADSCs cohort in comparison to the 'Unconverted HAP seeded ADSCs' cohort. The proteins listed are sorted with their respective trembl/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Protein Score	Coverage%	Role in Bone Morphogenesis
Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase	59	6.6	Procollagen-lysine,2-oxoglutarate 5-dioxygenase (PLOD-3) post transitionally modifies collagen through hydroxylation of lysine residues leading to the binding and cross-linking of fibrils to collagen fibers. This provides tensile strength to collagen fibers within the organic matrix of bone[378].
P10301	Ras-related protein R-Ras (R-Ras)	181	21.1	R-Ras promotes cellular adhesion through the regulation of the actin cytoskeleton [502]. Additionally, R-Ras has shown to interact with integrin beta-1 to mediate its cellular adhesion and migration capacity [503]. Additionally, R-Ras signaling has shown to induce osteogenesis in BMSCs [504].
Q02818	Nucleobindin-1	91	8.2	Nucleobindin 1 is a multifunctional calcium binding protein that was identified in the ECM of bone matrix and was later termed calnuc [374]. Within the bone matrix, calnuc binds and deposits calcium to form hard tissue. Additionally, calnuc binds hydroxyapatite to import calcium inside osteoblasts and osteocytes [375].
A0A0D9SFE5	Lamin B1, isoform CRA_a	116	25	Lamin B1 is a multifunctional nuclear intermediate filament that supports the structural integrity of the nucleus, and play a key role in stem cell niche function such as cell proliferation and differentiation of specific lineages, including bone formation [377]. A high ratio of lamin B is correlative to the stiffness of a cell and thus bone tissue is highly abundant in lamin A and lamin B [225]
P09525	Annexin A4			During skeletal development of annexins is well-characterized in bone as components of mineralizing matrix vesicles[300]. Annexin A4 is a calcium

				dependent phospholipid membrane binding protein modulates NF-κβ signalling by directly interacting with the p50 heterodimeric p50/65 NF-κβ complex which participates in the RANKL pathway of osteoclast regulation [301] [302].
O60763	General vesicular transport factor p115	81	6.5	GVTF-p115 is a membrane transport factor that translocates from the cytosol to the Golgi membrane during interphase to mediate the compartmentalization of secretory proteins [506]. It's participation in bone morphogenesis however is yet to be elucidated.
P30041	Peroxiredoxin-6			PDX6 is an enzyme that has both glutathione peroxidase and lysosomal type phospholipase A2 activities, and it also possess an antioxidant activity. The peroxidase family is heavily involved in the regulation of ROS activity within the body. The increase in peroxidase expression suggests it acts as a protective measure against ROS mediated tissue damage during osteoarthritis.[488]
O75369	Filamin-B			Filamin B cross-links actin which enables intracellular communication between the cytoskeleton and cell membrane during skeletal development. Various studies have shown that a mutation within the filamin B gene [380]
187114	Beta-galactoside-binding lectin, partial	127	35.8	During the matrix maturation stage of osteoblastic development, beta-galactoside-binding lectin was found to be upregulated in a study investigating the protein profiling of osteo-differentiated NSQ50 cell lines [514]
A0A0B4J1R6	Transketolase	65	6.1	Transketolase is an enzyme which regulates the pentose phosphate pathway in all organisms. It is found to be secreted during the early stages mesenchymal osteoblast differentiation however its significant role during osteogenesis is unknown [572].
P11532	Dystrophin			Dystrophin is a muscle cytoskeletal protein. Mutations in the dystrophin gene results in Duchenne Muscular Dystrophy (DMD) which leads to muscle and bone deterioration [573]. The mechanism through which osteo degradation occurs is subsequently explained in the discussion.
119595779	laminin, alpha 5, isoform			The expression of laminins on the surface of human mesenchymal stem cells plays an important role for cyto-adherence [319]. Specifically, *in vivo* and *in vitro* studies have shown laminin alpha 5 to recruit and adhere osteoblasts during osteodifferentiation[320] .

F1MAN8	calnexin, partial	86	13.6	Calnexin is a calcium binding protein commonly expressed within the microvesicles of osteoblast like cells, called Saos-2 cells. It's thought that calnexin is an important component in the mineralisation process of HA within the lumen of differentiating Saos-2 cells [324].
119613224	DEAH (Asp-Glu-Ala-His) box polypeptide 15, isoform			DEAH box proteins are a class of enzymes which heavily regulate post transcriptional modifications of RNA. Due to a large array of possible DEAH proteins within the human genome, it is thought the expression of one is indicative of a specialized role [520]. The function of DEAH (Asp-Glu-Ala-His) box polypeptide 15, isoform however, is unknown.
Q15149	Plectin	44	7	Plectin is a cytoskeletal cross linking protein which maintains mechanical integrity of many skeletal tissues, including muscle and bone. Additionally, the inhibition plectin has shown to bypass cellular senescence in rat fibroblasts [373].
Q92882	Osteoclast-stimulating factor 1	48	3.7	OsF-1is an intracellular protein that induces osteoclast formation and the resorption of inorganic hydroxyapatite. Additionally, OsF-1is produced by osteoclasts to mediate its bone homeostasis effects via signaling complexes with its diverse biological partners [523].
B3V096	Beta CstF-64 variant 2			BetaCstF-64 is involved in tissue specific polyadenylation which contributes to proteomic diversity to allow specialised functions of particular proteins [496]. Its role in osteogenesis is not known.
14250317	Similar to Plastin 3	56	7.9	Plastin 3 is a filamentous actin-binding protein bone matrixes and is regarded as a bone regulatory protein. Mutations within the Plastin-3 have shown to be associated with bone fractures and the formation of osteoporosis in humans [464]
P33897	ATP-binding cassette sub-family D member 1 (ABCD-1)	51	5.7	ABCD-1, also known as peroxisomal ABC transporter protein (ALDP) that catalyses medium chain fatty acids for transportation to and from the cytosol. The role of ABCD-1 in osteogenesis has not been identified [574].
Q8NCM8	Cytoplasmic dynein 2 heavy chain 1 (DYNC1H1)	55	4.3	The DYNC1H1is involved is a house keeping protein for neuron specific cellular processes. During mesenchymal development DYNCH1H1 has shown to be important for cell size sensing to regulate size homeostasis [575].
124504560	Flotillin-1			Flotillin-1 is a membrane protein that has been distributed exclusively in a lipid raft membrane fractions in various cells. During osteoclastogneogenesis, the

				raft component flotillin are increased after stimulation with macrophage colony stimulating factor [293].
Q13885	Tubulin beta-2A chain	830	41.6	Components of tubulin are a major constitute of cellular microtubules. The function of Tubulin beta 2A chain is unknown.
O43294	Transforming growth factor beta-1-induced transcript 1 protein	53	9.8	Transforming growth factor-β (TGF-β) plays an important role in bone metabolism by functioning as a coupling factor that links bone resorption and migration of bone mesenchymal stem cells to the remodeling area during osteogenesis [576].

Supplementary Table 4.9: Listed are some of the unique proteins identified from the 'Unconverted HAP seeded ADSCs' cohort in comparison to the 'Converted HAP seeded ADSCs' cohort. The proteins listed are sorted with their respective trembl/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
P62829	60S ribosomal protein L23	96	34..3	L23 is a regulator of MDM2 during ribosomal stress to regulate cell cycle proliferation [577].
P06702	Protein S100-A9	100	36.8	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell migration and adhesion [408]. Additionally. chondrocytes express S100A9 and their upregulation is associated with IL-1, suggesting a role in cartilage repair during inflammatory induced tissue damage[475].
NP_001138471.1	integrin alpha-V (IαV2) isoform 2 precursor (IαV2)			The alpha-V integrins are receptors for vitronectin, fibronectin, osteopontin, osteomodulin and matrix mettaloproteinase-2 (MMP-2) to facilitate skeletal tissue repair [476]. IαV2 under goes post-transitional processing to create multiple isoforms that bind to extracellular matrix protein
P05109	Protein S100-A8	59	26.9	Similarly to S100 A9, the S100A8 protein is a calcium binding protein that forms a complex with S100A9 to initiate NFκB and MAP kinase signaling which has shown top promote osteogenesis in MSCs [409] [410]. The role of S100A9 within the complex is to prevent S100A8 degradation as S100A is the active component of the complex [479].
A2A3R5	Ribosomal protein S6	47	21.3	Growth factors and mitogens such as insulin growth factor and epidermal growth factor (both of which identified) are known to activate S6k protein as it is thought to be a key regulator for cellular homeostasis. However, the sole function of ribosomal protein S6 has not been elucidated [578]

P37802	Transgelin 2	118	15.9	The function pf transgelin 2 is unknown however, Sekigawa *et al* found that transgelin 2 was upregulated within the serum/plasma of osteoarthritic patients during infliximab treatment [484]. Additionally, Elsafadi *et al* showed that the gene responsible. for the transgelin family proteins are important for the for osteoblastic differentiation of human mesenchymal stem cells as it regulates cytoskeleton arrangement and distribution of actin filaments [485] .

Supplementary Table 4.10*:* Listed are some of the unique proteins identified from the 'Macroporous seeded ADSCs' cohort in comparison to the 'Nanoporous ADSCs' and 'ADSC' cohort. The proteins listed are sorted with their respective trembl/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
Q9Y490	Talin-1	131	9.9	Talin-1maintains the actin cytoskeleton adhesion by complexing with various integrins. During osteoclast activation,Talin-1 binds to macrophage colony stimulating factor (MCSF) and β integrin to mediate proper cytoskeleton rearrangement to ensure osteoclast viability [381].
Q99623	Prohibitin-2	195	38.1	PHB2 modulates estrogen receptor alpha to regulate RunX2 transcription which accelerates and initiates osteoblastogenesis[539].
Q00610	Clathrin heavy chain 1	219	6.6	Clathrin is a trimer of heavy chains that mediate endocytosis for membrane protein trafficking. It is involved in the uptake of ligand receptor complexes, membrane transporters and adhesion molecules [353]
P81605	Dermcidin	72	17.3	Dermcidin has dual functions which include the 47 amino acid DCD-1 peptide that has shown to have antimicrobial activity[458] and the 30 amino acid PIF-CP peptide that mediates proteolytic activity [459]. The expression of PIF-CP occurs during mitochondrial stress which is initiated by calcium extrusion pathways at the plasma membrane [460]
P59998	Actin-related protein 2/3 complex subunit 4 (Arp 2/3 complex)	46	14.3	Arp 2/3 complex protein activates actin filaments to maintain multiple forms of actin based structures that occur during the constant rearrangement of cytoskeletal framework of cells during cellular differentiation and migration[364] [365]. It's specific role in osteo differentiation however has not been elucidated.

P50990	T-complex protein 1 subunit theta (TCP1-θ)	160	21.7	The theta subunit of the chaperone protein which is involved in the folding of actin and tubulin after high metabolic activity such as ATP hydrolysis [450] A study investigating the potential biomarkers for osteonecrosis of the jaw found TCP1-θ to be downregulated in osteonecrotic patients [451].
P49411	Elongation factor Tu, mitochondrial (Ef-Tu)	84	6.4	Ef-Tu is a mitochondrial protein which promotes the binding of aminoacyl tRNA in ribosomes during protein synthesis.
P35232	Prohibitin	178	44.5	Prohibitin is a multi-functional membrane protein that has shown to increases cellular migration capacity and proliferation in mesenchymal stem cells [447].
P31947	14-3-3 protein sigma	85	16.9	An isoform of the 14-3-3 protein family of proteins, 14-3-3 protein sigma is unique in a sense as it has found to be expressed in osteo-articular cells during induced compression highlighting a role in the maintenance of articular osteoblasts during chronic compression [564]
P19367	Hexokinase-1	90	11.4	Hexokinase-1 phosphorylates glucose to synthesise glucose-6-phosphate for the production NADPH during high energy metabolic events such as cellular differentiation in pluripotent stem cells [545] .
P19105	Myosin regulatory light chain 12A (MYL12A)	92	26.9	MYL12A is a calcium binding myosin regulatory subunit that maintains the stability of myosin II for ECM integrity and additionally, plays an important role in regulation of both smooth muscle and nonmuscle cell contractile activity [579] [580].
P06733	Alpha-enolase	240	33.9	Enolase 1 is a multifunctional protein that is induced by pro-inflammatory stimuli and expressed during cellular differentiation [548]. Additionally, it has found to be overexpressed in rheumatoid arthritis fibroblasts -like synoviocytes and play a role in glycolysis [549].
P06703	Protein S100-A6 (Calcyclin)	63	8.9	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell adhesion and differentiation [369]. Protein S100-A6, also known as calcyclin is responsible for sensing extracellular calcium and other cations such as aluminum and magnesium to regulate the osteoblast function [370].
P04083	Annexin A1	330	23.7	Annexin A1 binds to the cellular membrane in calcium dependent manner in a similar fashion as the other annexins from the same superfamily of proteins [537]. The molecular function of annexin 1 ranges from regulating the cytoskeletal rearrangement of MSCs and osteoblasts during osteogenesis and

				to inhibit spontaneous differentiation of MSC populations[538].
P01023	Alpha-2-macroglobulin	77	9.4	Alpha-2-macroglobulin is a protease which inhibits a number of proteolytic enzymes including metalloproteinases which are responsible for the degradation of ECM within skeletal tissue [468]. Additionally, alpha-2-macroglobulin is known to bind to a number of growth factors and cytokines including TFG-β and FGF [469].
NP_001138471.1	integrin alpha-V isoform 2 precursor (IαV-2)	68	4.6	The alpha-V integrins are receptors for vitronectin, fibronectin, osteopontin, osteomodulin and matrix mettaloproteinase-2 (MMP-2) to facilitate skeletal tissue repair [476]. IαV-2 under goes post-transitional processing to create multiple isoforms that bind to extracellular matrix protein
G3V3E6	ERO1-like protein alpha	231	18.6	ERO1-like protein alpha is an oxireductase enzyme which is heavily involved in disulfide bond formation of synthesised protein within the endoplasmic reticulum [473].
C9J5W0	Translocon-associated protein subunit alpha	63	7.4	TRAP-α is part of a complex which binds calcium to the endoplasmic reticulum membrane to regulate the retention time of ER resident proteins. Due to its ER location, it may be involved in the translocation process of protein folding and recycling
B7Z9L0	T-complex protein 1 subunit delta	61	12.2	T-complex protein 1 subunit molecular chaperone; assists the folding of proteins upon ATP hydrolysis. Known to play a role, *in vitro*, in the folding of actin and tubulin.
B4DHZ6	Transferrin, isoform CRA_c	65	6.5	Transferrin is a glycoprotein which can readily bind iron and in the absence of iron is called apoprotein which interacts with insulin like growth factors (IGF) to modulate IGF functions such as osteogenesis and chondrogenesis[492] [493].
O96019	Actin-like protein 6A (ACTL6A)	841	54.9	ACTL6A alters the DNA nucleosome topology to suppress selective genes and is also involved in transcriptional activity [356]. Additionally, ACTL6A was found be expressed by somatic adult precursor cells to control nucleosome associated differentiation [357].
Q92743	Serine protease (HTRA1)	78	10.7	HTRA1 degradation of specific glycoproteins within the skeletal ECM has shown to play a central role in controlling mineral deposition by osteoblasts [321]. Importantly, the serine protease has been found to be a positive regulator of mesenchymal stem cell osteogenesis as the expression of HTRA1

				increased in a time dependent manner [327].
229002090	CD44 molecule	69	8.6	CD44 is a multifunctional adhesion molecule that binds to hyaluronic acid. type 1 collagen, and fibronectin. It is found to be expressed on the plasma membrane of differentiating osteocytes as it is a core component of ECM regulation during bone morphogenesis[361].
Q8WXE9	Stonin-2	60	3.8	Stonin 2 is an endocytic protein that interacts with the clathrin/AP-2 complex as an AP-2 dependent sorting adaptor protein which targets synaptotagmins for endocytic internalisation[354]. Synaptotagmins enable calcium-induced exocytosis of essential ECM molecules in differentiating osteoblasts[355].
P13929	Beta-enolase	58	4.9	Enolase 3 is a glycolytic enzyme that innervates skeletal striated muscle during contraction and regenerates muscle tissue after fatigue [465] [466]. Beta enolase is a known marker for precursor myoblasts however its presence and role in bone morphogenesis Is unknown [467].
19607750	Fascin homolog 1, actin-bundling protein	107	18.5	Fascin is an actin binding protein which colocalises actin filaments to the periphery of the cell to meditate cell adhesion [366]
119593267	Biglycan, isoform CRA_a	59	10.1	Biglycan extracellular proteoglycan that positively regulates bone formation and osteoclast differentiation through its effects on osteoblasts and their precursors [382].
Q9UQ80	Proliferation-associated protein 2G4 (PA2G4)	56	4.4	PA2G4 is a RNA binding protein that regulates cell growth through its regulation of late and intermediate steps of RNA processing [581]. Its role in osteogenesis has not been highlighted.
Q14974	Importin subunit beta-1	82	5.7	Importin transports proteins into nucleus either through an association with an importin alpha subunit as a carrier protein or autonomously through the importin beta subunit through a Ran-dependent system. The process of protein transport into the nucleus is vital for particularly growth factors and cytokines to drive transcription and directed activity[354]
Q09666	Neuroblast differentiation-associated protein AHNAK	75	2.7	NdapA is a chaperone regulatory protein whose molecular function is unknown. However, studies have implicated the protein to play a role in osteoblasts as it is upregulated during endothelial-1 treatment of calvarial cells and osteoblast Saos-2 cell lines [486] [487].
P60981	Destrin	75	20	Destrin regulates disassembly and assembly of actin filaments which are essential for cellular membrane ruffling and extension, a process common in

				osteoblast differentiation into osteocytes during bone development[322].
P49588	Alanine-tRNA ligase, cytoplasmic	48	8.1	Alanine-tRNA ligase is responsible for the attachment of alanine to tRNA during the process of protein translation. Additionally, Alanine-tRNA ligase has an editing domain which edits incorrectly charged tRNA. To date, there is no study which has linked Alanine-tRNA ligase to bone morphogenesis [463].
P48552	Nuclear receptor-interacting protein 1 (NRIP1)	110	2.3	NRIP1 mediates the transcriptional activation of steroid receptors that are similar to nuclear receptor isomers of NR3C1 and ESR1 [582]. Additionally, NRIP1 modulates transcriptional repression by nuclear hormone receptors [583].
P41252	Isoleucine--tRNA ligase, cytoplasmic	126	19.7	Isoleucine-tRNA ligase is an enzyme that activates its substrates 1 isoleucine and L valine during its first amino-acylation step process [584]
P40227	T-complex protein 1 subunit zeta	68	17.1	A molecular chaperone that assists in the folding of actin and tubulin *in vitro*[367], The proper folding of actin and tubulin is essential for many biological processes however its role in osteogenesis has not been elucidated.
P38919	Eukaryotic initiation factor 4A-III (EIF4A3)	76	19.5	EIF4A3 is involved in RNA metabolism and plays an important role in MRNA decay, splicing and translation. The loss of EIF4A3 leads to a loss of cranial neural cells (CNC) and results in skeletal manifestations of Richieri-Costa-Pereira syndrome (RCPs) [585].
P35749	Myosin-11	429	17.9	Myosin-11 is a hexameric contractile protein that converts the energy generated from ATP hydrolysis into mechanical contraction within smooth muscle tissue. Additionally, myosin 11 participates in the stabilisation of osteoclast lamellipodium which allows the osteoclasts to resorb substrate within the lacunae of bone [388].
P11047	Laminin subunit gamma-1 (Lγ-1)	100	5.9	Lγ-1 binds to skeletal ECM components with high affinity to mediate attachment and organisation of neighboring cells. Additionally, Lγ-1, Lα-1 and Lβ-1 has been found to be differentially expressed during osteogenesis [368]
K7EJ44	Profilin 1, isoform CRA_b	122	11.2	Profilin- 1 is a ubiquitously expressed G-actin protein that promotes actin polymerisation. It is essential for actin reorganization during osteoclast bone resorption [383]. Additionally, profilin is highly upregulated during BMSCs osteodifferentiation [303].
I3L504	Eukaryotic translation initiation factor 5A-1	89	15.1	Eukaryotic translation initiation factor 5A-1 was identified as a critical protein involved in osteogenic differentiation of umbilical cord blood mesenchymal

				stem cells due to its 10 fold increase during induced bone morphogenesis[455].
B4DW73	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	75	11.3	Phosphoenolpyruvate carboxykinase is a gluconeogenic enzyme that is expressed in variety of human tissues such as the pancreas and fibroblasts as it is involved in glyceroneogenesis [455].
B4DSD8	Alpha-1,4 glucan phosphorylase	59	7.2	Alpha-1,4 glucan phosphorylase releases alpha-D-glucose 1-phosphate by severing the alpha 1,4 link between pairs of glucose molecules at the end of long glucose polymers. This phosphorylases activity allows essential phosphorylation of glucose residues and thus plays a role in carbohydrate metabolism[482,483].

Supplementary Table 4.11*:* Listed are some of the unique proteins identified from the 'Nanoporous seeded ADSCs' cohort in comparison to the 'Macroporous seeded ADSCs' and 'ADSC' cohort. The proteins listed are sorted with their respective trembl/nniprot accession number, protein score, '%' coverage and a brief description of their role in bone morphogenesis.

Accession	Protein Name	Transmembrane (Y/N)	Coverage% (#unique peptides)	Role in Bone Morphogenesis
Q7RTY7	Ovochymase-1	55	1.4	Ovochymase-1 is a chymotrypsin like protease which has been characterized to play a role in oocyte development and was found to be exclusively secreted onto the cell surface [494].
Q15149	Plectin	57	3.8	Plectin is a cytoskeletal cross linking protein which maintains mechanical integrity of many skeletal tissues, including muscle and bone. Additionally, the inhibition plectin has shown to bypass cellular senescence in rat fibroblasts [373].
P26447	Protein S100-A4	63	8.9	S100A4, also known as calvasculin, a calcium binding protein that interacts with microtubilin, actin and myosin to regulate cytoskeletal dynamics and elements such as cellular proliferation[371]. Interestingly, S100A4 has known to upregulate MMP-13 to stimulate chondrocyte differentiation [372].
P06703	Protein S100-A6	65	8.9	S100 proteins are intracellular calcium binding proteins that control key cellular pathways including regulation of cell adhesion and differentiation [369]. Protein S100-A6, also known as calcyclin is responsible for sensing extracellular calcium and other cations such as aluminum and magnesium to regulate the osteoblast function [370].
NP_001138471.1	integrin alpha-V isoform 2 precursor	68	4.6	The alpha-V integrins are receptors for vitronectin, fibronectin, osteopontin, osteomodulin and matrix mettaloproteinase-2 (MMP-2) to facilitate skeletal tissue repair [476]. IαV2 under goes post-transitional processing to create multiple isoforms that bind to extracellular matrix protein

G3V3E6	ERO1-like protein alpha	113	9.2	ERO1-like protein alpha is an oxireductase enzyme which is heavily involved in disulfide bond formation of synthesised protein within the endoplasmic reticulum [473]. .
Q02818	Nucleobindin-1	91	8.2	Nucleobindin 1 is a multifunctional calcium binding protein that was identified in the ECM of bone matrix and was later termed calnuc [374]. Within the bone matrix, calnuc binds and deposits calcium to form hard tissue. Additionally, calnuc binds hydroxyapatite to import calcium inside osteoblasts and osteocytes [375].
B3V096	BetaCstF-64 variant 2	60	9.9	BetaCstF-64 is involved in tissue specific polyadenylation which contributes to proteomic diversity to allow specialised functions of particular proteins [496]. Its role in osteogenesis is not known.
A0A0D9SFE5	Lamin B1	48	5.9	Lamin B1 is a multifunctional nuclear intermediate filament that supports the structural integrity of the nucleus, and play a key role in stem cell niche function such as cell proliferation and differentiation of specific lineages, including bone formation [377]. A high ratio of lamin B1 is correlative to the stiffness of a cell and thus bone tissue is highly abundant in lamin A and lamin B [225].
P52907	F-actin-capping protein subunit alpha-2 (CapZA2)	86	9.4	CapZA2 is a F-actin capping protein CapZA2 binds the barbed end of actin filaments and prevents addition or loss of actin monomers to filaments in c calcium dependent manner which is why it is commonly expressed in skeletal muscle [497].
7709904	calnexin, partial	86	16.6	Calnexin is a calcium binding protein commonly expressed within the microvesicles of osteoblast like cells, called Saos-2 cells. It's thought that calnexin is an important component in the mineralisation process of HA within the lumen of differentiating Saos-2 cells [324].
Beta-enolase	Beta enolase	240	33.9	Beta enolase is a glycolytic enzyme that innervates skeletal striated muscle during contraction and regenerates muscle tissue after fatigue [465] [466]. Beta enolase is a known marker for precursor myoblasts however its presence and role in bone morphogenesis Is unknown [467].
P30048	Peroxiredoxin 3	70	45.4	PDX-3 is a redox regulator that protects the cell during high oxidative stress events such as cellular proliferation and differentiation. PDX-1 and PDX-5 have shown to be upregulated during osteogenesis however the role of PDX-3 during bone morphogenesis has not been revealed [470]..

Supplementary Table 4.12: The secretion profile of seeded converted cHA obtained by Bioplex analysis over 30 days. The reported values of secretion levels are sorted by relative concentration to the control conditioned hADSC medium. All values are means of duplicate samples (expressed as pg/ml).

		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30
Very low	**Hu IL-4 (52)**	15.48	1.72	5.36	0.96	11.48	1.16	5.36	3.32	5.2	5.36
	Hu IL-5 (33)	7.96	0	0	0	7.2	0	0	0	0	0
	Hu IL-13 (51)	15.68	3.08	12.96	2.76	6.16	0	0	0	0	0
Low	**Hu IL-1b (39)**	26.76	2.72	5.92	0.12	11.08	0	5.76	4.48	5.2	4.68
	Hu IL-2 (38)	44.32	13.24	23.4	7.88	9.24	10.64	7.12	13.64	9.84	11.04
	Hu IL-7 (74)	22.36	1.36	16.64	1.36	9.4	0	0	0	0	0
	Hu IL-9 (77)	32.96	8.4	11.96	8.92	21.88	5.92	8.92	10.68	9.4	9.4
	Hu IL-10 (56)	141.72	27.76	128.36	29.84	22.6	11.68	8.36	16.56	8.84	11.68
	Hu IL-12(p70) (75)	260.92	22.64	308.96	20.68	25.6	5.2	11.32	10.4	11.32	12.2
	Hu IL-15 (73)	94.36	33	59.88	14.92	11.64	28.6	12.6	29.8	18.92	25.72
	Hu Eotaxin (43)	96.36	9.88	37.84	0	44.2	0	29.92	27.24	27.24	25.88
	Hu IFN-g (21)	554.2	45.8	249.6	0	127.36	0	55.6	45.8	45.8	55.6
	Hu IP-10 (48)	97.56	15.68	36.36	12.12	86	0	50.76	37.56	39.88	39.88
	Hu MIP-1a (55)	19.96	14.36	4.72	1.76	18.68	0.28	10.84	7	8.8	7.36
	Hu PDGF-bb (47)	117.68	12.88	30.76	9.2	46.48	9.2	20.12	16.52	20.12	20.12

	Hu MIP-1b (18)	26.16	11.2	8.44	5.96	14.16	4.88	8.44	7	7.6	7.88
	Hu RANTES (37)	109.36	40.12	51.04	17.24	58.84	13.44	44.08	47.92	46.68	44.08
	Hu TNF-a (36)	385.76	24.72	129.8	0	138	0	63.2	47.08	50.32	43.88
Medium	**Hu G-CSF (57)**	1681.68	66.8	91.68	9.96	33.08	0	9.96	9.96	9.96	13.52
	Hu MCP-1(MCAF) (53)	2143.16	83.36	487.72	87.56	57.56	69.2	44.12	67.76	43.36	63.44
	Hu VEGF (45)	1493.52	82.68	1076.52	56.24	57.24	14	20.24	14	14.88	16.64
High	**Hu IL-6 (19)**	24664.72	959.24	8126.36	118.08	7.72	4.56	3.04	4.56	3.52	90.56
	Hu IL-8 (54)	11799.88	2561.12	3178.08	191.68	9.2	31.08	0	0	0	25.04
	Hu IL-17A (76)	1003.52	37.08	48.68	32.16	982.84	30.52	627.8	556.84	606.52	602.96
	Hu FGF basic (44)	773.4	36.12	73.12	68.44	1357.68	57.16	811.96	444.4	617.64	568.2

Supplementary Table 4.13: The secretion profile of seeded unconverted coralline calcium carbonate obtained by Bioplex analysis over 30 days. The reported values of secretion levels are sorted by relative concentration to the control conditioned hADSC medium. All values are means of duplicate samples (expressed as pg/ml).

		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30
Very low	**Hu IL-5 (33)**	0	0	0	0	0	0	0	0	0	0
	Hu IL-7 (74)	14.28	17.8	0	23.48	0	0	0	4.24	0	0
Low	**Hu IL-1b (39)**	40.28	6.28	0	6.84	0	0	4.68	7.4	4.84	5.2
	Hu IL-2 (38)	25.32	20.44	0	18.76	6.72	32	12.24	17.96	14.64	11.84
	Hu IL-4 (52)	7.68	6.2	1.56	6.04	0	1.72	4.52	6.44	3.84	5.88
	Hu IL-9 (77)	18.72	12.48	6.4	10.4	0	14	10.4	12.48	10.92	8.92
	Hu IL-10 (56)	70.6	100.84	30.92	144.16	12.64	45.96	15.08	38.36	16.56	16.56
	Hu IL-13 (51)	9.88	12.96	3.92	17.68	0	4.52	0	3.92	0	2.76
	Hu IL-15 (73)	59.16	51.2	0	46.04	20.64	112.24	24.04	40	32.2	22.36
	Hu Eotaxin (43)	47.36	37.84	6.64	45.48	0	8.32	27.24	44.2	27.24	27.92
	Hu IP-10 (48)	42.16	37.56	12.12	39.88	0	13.96	39.88	49.72	42.16	54.88
	Hu MIP-1a (55)	277	4	8.6	3.72	0	0.8	7.64	9.24	7.76	7.2
	Hu PDGF-bb (47)	48.2	32.52	7.36	32.52	5.48	23.68	20.12	29	21.88	21.88
	Hu MIP-1b (18)	137.24	9	4.88	7.28	3.48	10.96	8.72	10.68	9	9
	Hu RANTES (37)	229	50.44	15.36	51.68	3.96	35.32	50.44	60	51.68	54.08

	Hu TNF-a (36)	174.04	134.72	21.52	144.52	0	0	47.08	92.36	47.08	56.76
Medium	**Hu IL-1ra (25)**	1005	428.2	75.52	428.2	0	75.52	118.16	238.16	118.16	139.76
	Hu IL-12(p70) (75)	149.96	251.2	36.72	439.88	0	17.8	11.32	57.92	13.12	17.8
	Hu IL-17A (76)	82.08	52.8	28.88	42.04	19.12	47	572.8	633.16	588.76	546.24
	Hu FGF basic (44)	102.4	77.72	52.16	66.88	52.16	98.92	573.4	577.68	555.68	557.84
	Hu G-CSF (57)	1090.68	159.08	24.2	68.6	0	15.28	11.76	29.52	13.52	72.12
	Hu GM-CSF (34)	463.36	324.28	294.64	222.84	318.28	718.2	316.04	337.4	332.52	269.88
	Hu IFN-g (21)	300.12	256.08	35.44	281.48	0	11.24	45.8	138.8	45.8	82.72
	Hu MCP-1(MCAF) (53)	171.64	594.68	84.76	715.2	70.64	164.6	56.12	298.52	76.32	109.48
	Hu VEGF (45)	551.36	982.08	133.96	1786.88	7.2	25.72	14.88	285.88	19.32	54.28
High	**Hu IL-6 (19)**	12720.04	8824	467.52	9390.76	0	0	0	3385.92	35	1282.92
	Hu IL-8 (54)	20353.08	5689.8	985.08	1913.04	28.04	21.56	0	277.12	18.08	444.8

Supplementary Table 4.14: The secretion profile of seeded macroporous cHA obtained by Bioplex analysis over 30 days. The reported values of secretion levels are sorted by relative concentration to the control conditioned hADSC medium. All values are means of duplicate samples (expressed as pg/ml).

		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30
Very low	**Hu IL-5 (33)**	9.44	0	4.72	0	0	0	0	0	0	0
Low	**Hu IL-1b (39)**	26	9.6	26.48	5.04	0	0	0	5.04	14.16	5.56
	Hu IL-2 (38)	49.28	15.28	34.16	20.44	11.04	5.2	11.04	1.92	41.2	11.04
	Hu IL-4 (52)	16.12	2.8	8.32	4.96	1.16	1.16	0.6	6.28	9.12	5.72
	Hu IL-7 (74)	36.64	4.24	22.36	16.64	0	0	0	0	6.88	2.84
	Hu IL-9 (77)	26.72	9.4	32.12	10.92	4.96	4.96	4.48	7.4	29.4	7.92
	Hu IL-10 (56)	247.36	33.04	193.84	113.32	16.56	22.6	11.68	0	90.24	28.28
	Hu IL-13 (51)	26.52	6.16	19.12	13.96	0.92	1.56	1.24	0.92	6.72	3.36
	Hu IL-15 (73)	208.56	36.92	82.04	50.48	29	7.16	34.56	0	77.64	20.64
	Hu Eotaxin (43)	100.84	18.88	51.72	37.84	3.16	1.16	1.16	24.48	65.08	31.28
	Hu IP-10 (48)	108.64	24.84	48.68	35.16	12.12	8	8	46.52	184.2	35.16
	Hu MIP-1a (55)	33.6	41.72	190.36	2.96	0.28	0.44	0	8.84	16.76	7.12

	Hu PDGF-bb (47)	89.16	16.52	65.4	29.88	5.48	7.36	7.36	12.88	60.24	18.32
	Hu MIP-1b (18)	22.92	19.6	55.48	7.28	4.28	4.28	3.96	5.8	20.24	7
	Hu RANTES (37)	416	138	121.28	44.08	15.36	0	11.36	27.24	110.32	46.68
	Hu TNF-a (36)	392.4	47.08	183.88	115.12	8.92	8.92	5.8	50.32	174.04	69.64
Medium	**Hu FGF basic (44)**	165.48	73.12	170.96	79.2	63.68	70.8	65.28	738.36	861.56	476.4
	Hu IL-12	742.8	48.24	353.24	289.08	9.52	8.64	6.88	7.76	66.68	48.24
	Hu IL-17A (76)	149.96	40.4	109.92	45.36	25.64	29.72	19.12	555.08	892.12	480.84
	Hu G-CSF (57)	8134	262.84	334.64	64.16	6.4	8.2	4.64	17.08	271.68	22.4
	Hu GM-CSF (34)	470.84	388.68	844.72	301.8	322.8	369.6	337.4	292.4	662.36	204.76
	Hu IFN-g (21)	621.56	107.68	306.28	223.36	0	0	0	45.8	230	99.56
High	**Hu IL-1ra (25)**	1312.16	282.48	1240.92	338.24	23.6	13.56	3.8	64.96	507.56	172.32
	Hu IL-6 (19)	41959.8	2823.32	9457.88	6975.48	10.92	3.28	4.04	84.8	5449.12	2267.56
	Hu IL-8 (54)	31269.2	7906.08	268971	1818.92	94.4	88.04	35.52	23.88	2084	176.4

	Hu MCP-1(MCAF)	4897.92	104.04	298.52	521.36	80.56	93.08	70.64	34.84	388.64	230.96
	Hu VEGF (45)	3349.8	190.2	1249.84	1148.2	11.4	14.88	6.36	14.88	216.96	243.44

Supplementary Table 4.15: The secretion profile of seeded nanoporous cHA obtained by Bioplex analysis over 30 days. The reported values of secretion levels are sorted by relative concentration to the control conditioned hADSC medium. All values are means of duplicate samples (expressed as pg/ml).

		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30
Very low	**Hu IL-5 (33)**	4.72	0.36	0	0	0	0	2.84	0.36	0	0
Low	**Hu IL-1b (39)**	40.84	11	1.64	0	0	5.4	8.32	10.32	4.48	4.48
	Hu IL-2 (38)	40.32	27.44	0.6	12.64	11.84	8.28	37.04	27.68	13.04	4.84
	Hu IL-4 (52)	10.84	8.16	1.56	0.96	1.16	4.88	7.68	7.52	3.68	5.52
	Hu IL-7 (74)	26.28	25.72	0	0	0	20.68	26.84	13.08	0	0
	Hu IL-9 (77)	24.28	18.72	4.48	5.92	6.4	8.4	17.12	19.76	9.92	7.4
	Hu IL-10 (56)	172.56	161.36	17.56	16.56	21.56	127.12	173.8	106.76	13.6	11.68
	Hu IL-13 (51)	18.88	20.32	2.16	0.92	2.16	15.96	17.92	10.92	0.92	0.92
	Hu IL-15 (73)	84.72	57	0	39.24	31	9.2	110	57.36	25.72	3.32
	Hu Eotaxin (43)	68.64	48	1.16	3.16	1.16	41.68	59.64	54.16	24.48	18.88
	Hu IP-10 (48)	54.88	50.76	8	8	15.68	30.2	48.68	48.68	35.16	36.36
	Hu MIP-1a (55)	235.28	45.08	2.64	0.56	0.56	2.96	4.72	11.72	6.72	6.36
	Hu PDGF-bb (47)	68.8	48.2	3.6	9.2	7.36	27.24	49.92	34.28	16.52	9.2
	Hu MIP-1b (18)	144.76	20.48	3.96	3.32	5.2	5.8	11.48	11.48	8.44	7

	Hu RANTES (37)	103.72	72.36	46.68	17.24	17.24	34.64	73.44	72.36	45.4	30.28
	Hu TNF-a (36)	246.48	172.4	12.08	8.92	8.92	121.64	206.92	157.64	43.88	47.08
Medium	**Hu IL-12(p70) (75)**	441.96	493.32	13.12	7.76	9.52	333	449.64	190.04	12.2	10.4
	Hu IL-17A (76)	112.48	70.32	20.76	24	32.16	33.8	67	553.32	563.96	618.92
	Hu GM-CSF (34)	422.44	389.4	331.76	314.16	393.16	234.16	451.44	317.52	292.8	303.28
	Hu IFN-g (21)	429.28	336.6	11.24	0	0	243.12	324.56	209.92	35.44	45.8
	Hu MCP-1(MCAF) (53)	555.92	394.4	70.64	79.16	101.32	589.16	955.28	523.6	54.64	56.12
High	**Hu IL-1ra (25)**	928.88	587.48	44.08	13.56	13.56	349.44	576	439.48	107.44	64.96
	Hu IL-6 (19)	19889.72	11972.36	307.4	20.4	8.8	7826	10919.28	5490.48	4525	3269.32
	Hu IL-8 (54)	20722.64	16909.84	1583.44	126.88	43.64	1657.72	2487.56	575.84	3.04	0
	Hu G-CSF (57)	2132.44	306.28	27.72	6.4	9.96	57.92	97.88	52.6	8.2	11.76
	Hu VEGF (45)	2087.96	2077.76	45.04	12.28	21.16	1307.76	1708.36	816.72	14	10.56

Supplementary Table 4.16: The secretion profile of seeded High density cHA obtained by Bioplex analysis over 30 days. The reported values of secretion levels are sorted by relative concentration to the control conditioned hADSC medium. All values are means of duplicate samples (expressed as pg/ml).

		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30
Very low	**Hu IL-5 (33)**	9.44	0.36	0	0	0	0	0	0.36	0	0
Low	**Hu IL-1b (39)**	80.52	33.68	1.84	0.64	0.32	0	13.32	7.2	4.12	11.8
	Hu IL-2 (38)	37.92	26.4	5.56	27.24	20.44	10.24	30.68	12.24	11.04	27.88
	Hu IL-4 (52)	13.04	8	2.28	1.92	1.72	1.16	8.4	7.52	3.84	9.76
	Hu IL-7 (74)	21.24	14.28	4.24	4.24	5.56	0	5.56	1.36	0	4.24
	Hu IL-9 (77)	28.88	19.24	6.4	22.44	18.72	4.96	15.04	10.92	9.92	22.44
	Hu IL-10 (56)	125.92	80.96	30.92	99.64	73.48	18.56	29.84	17.56	13.6	38.36
	Hu IL-12(p70) (75)	270.72	178.2	39.84	22.64	33.64	8.64	46.12	29.56	12.2	35.16
	Hu IL-13 (51)	15.44	10.12	3.92	6.72	6.16	1.56	4.52	2.76	1.24	4.52
	Hu IL-15 (73)	82.04	47.52	8.72	107.08	62	33	70.4	24.04	20.64	52.68
	Hu Eotaxin (43)	74.52	46.12	8.32	21.72	9.88	1.16	62.68	37.84	27.24	49.24
	Hu IP-10 (48)	60.8	42.16	15.68	15.68	18.96	2.4	92.68	231.4	37.56	167.16
	Hu MIP-1a (55)	397.16	242.56	7.36	1.76	2.12	0.56	14.96	11.28	7.24	16.12
	Hu PDGF-bb (47)	77.32	46.48	11.04	43	23.68	5.48	49.92	27.24	16.52	44.72

	Hu MIP-1b (18)	219.92	115.28	7.44	12.84	10.12	4.88	18.24	13.08	7.28	18.76
Medium	**Hu IL-17A (76)**	134.6	79.56	24	55.32	50.32	20.76	674.96	528.56	542.68	987.32
	Hu FGF basic (44)	306.72	116.16	62.08	147.48	112.08	73.12	580.24	649.84	536.92	1050.16
	Hu IFN-g (21)	457.16	293.96	55.6	24.16	35.44	0	287.72	146.28	45.8	175.32
	Hu MCP-1(MCAF) (53)	328.68	224.8	79.16	207.48	157.52	83.36	787.24	338.08	56.12	214.92
	Hu RANTES (37)	627.04	191.28	19.04	37.4	24.08	11.36	86	71.8	45.4	85.48
	Hu TNF-a (36)	267.96	160.92	31.08	27.88	24.72	5.8	197.04	95.6	40.68	141.24
	Hu VEGF (45)	1307.76	783.52	162.12	43.6	78.16	13.16	188.56	104.2	15.76	94.4
High	**Hu IL-6 (19)**	28090.92	11065.48	1326.32	14.68	81.96	14.68	12687.8	4482.08	3.52	3523.32
	Hu IL-8 (54)	39521.92	19789.88	2121.08	257.48	218.52	21.2	2100.88	832.24	0	832.68
	Hu G-CSF (57)	3766.36	733.44	82.8	25.96	18.84	6.4	526.96	285	9.96	203.44
	Hu GM-CSF (34)	965.28	508.88	304.04	868.52	630.72	382.68	369.96	234.16	302.92	373.32

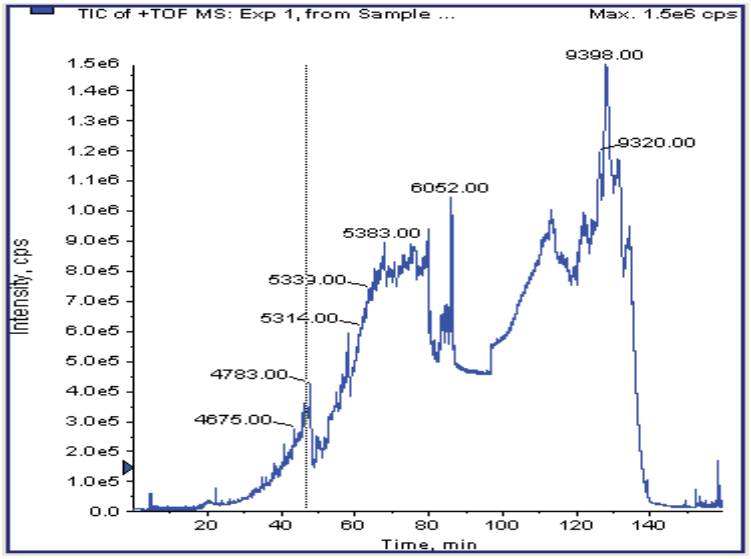

Figure S5.1a: Annotated LC-MS/MS TIC of seeded macroporous cHA semi-tryptic

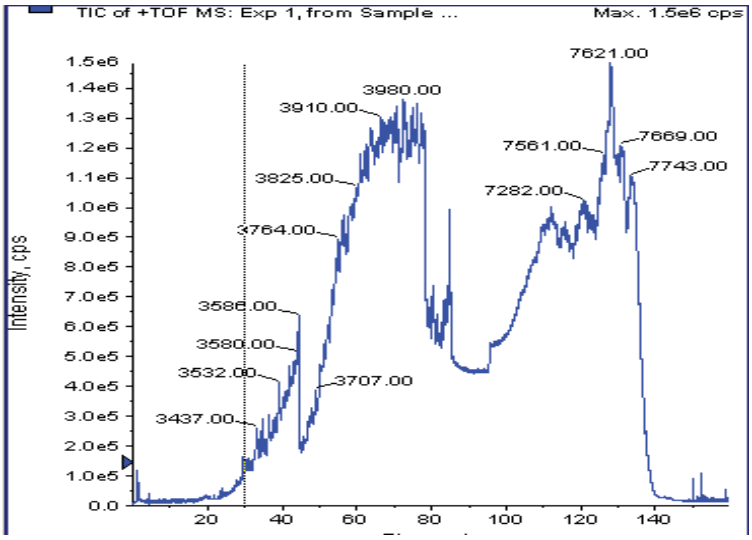

Figure S5.1b: Annotated LC-MS/MS TIC of seeded nanoporous cHA semi-tryptic

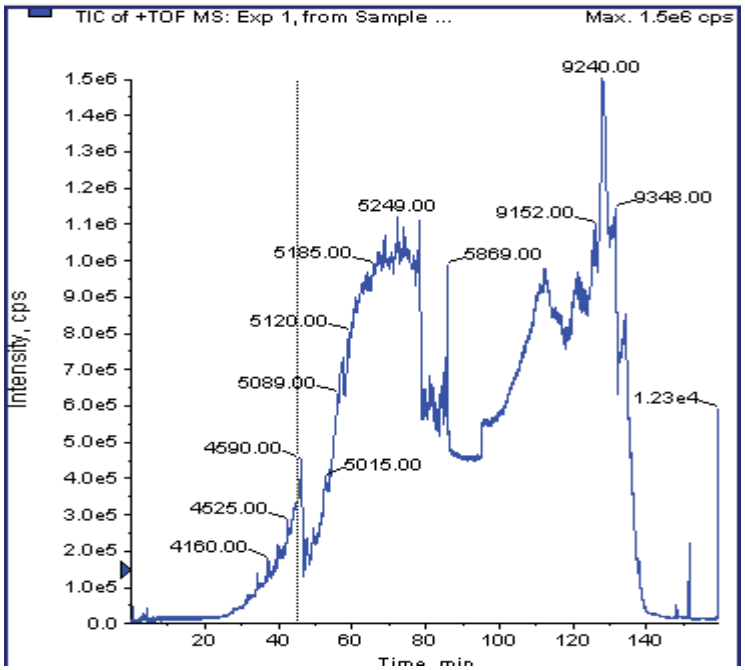

Figure S5.1c: Annotated LC-MS/MS TIC of seeded macroporous cHA semi-tryptic

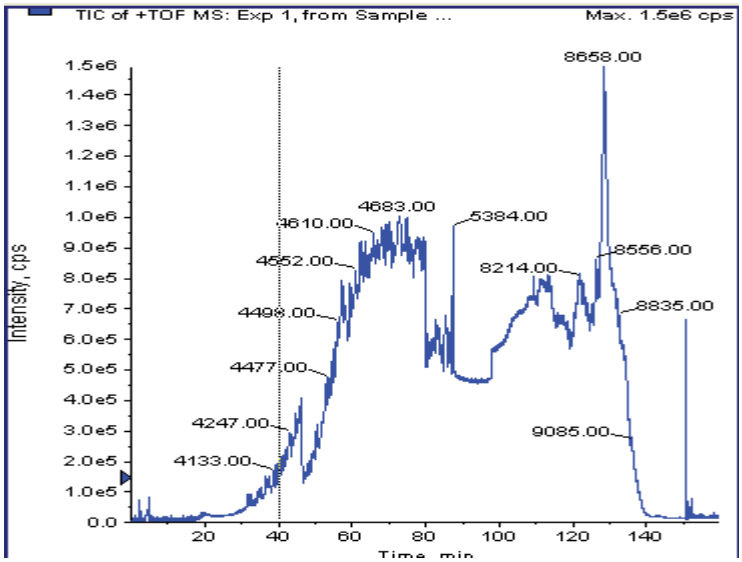

Figure S5.1d: Annotated LC-MS/MS TIC of seeded unconverted cHA semi-tryptic

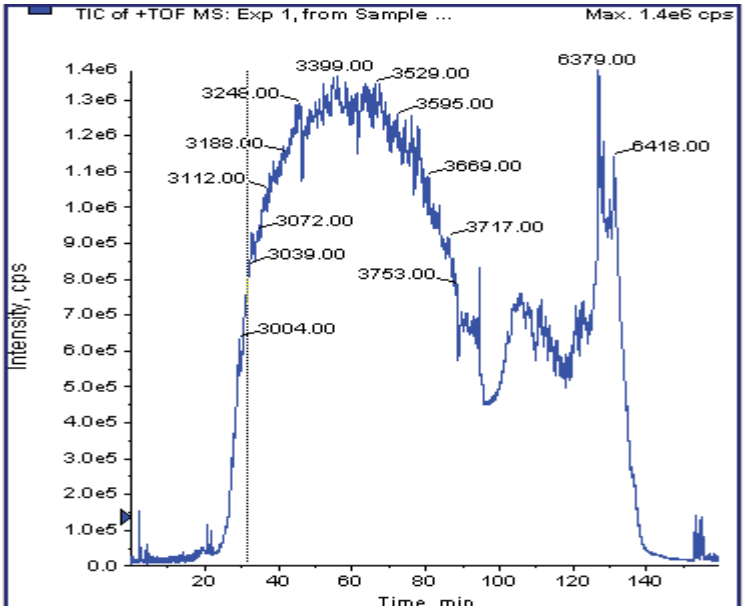

Figure S5.1e: Annotated LC-MS/MS TIC of seeded high density cHA semi-tryptic

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