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IDENTIFICATION OF IN SITU PROGENITOR CELL FEATURES CONTRIBUTING TO CARTILAGE REPAIR AND STRATEGIES FOR CARTILAGE REPAIR AUGMENTATION

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DEDICATION

To my husband,

My brother

&

My parents

Thanks for all the support and inspiration

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It was summer of 2012 and I was new and slightly apprehensive in a new country, trying to adapt to an unfamiliar environment. At this point, my research project provided me the stability and purpose I needed. For this, I thank my Ph.D. research supervisor, Professor Michael D Buschmann for providing me the opportunity to expand my learning in the beloved field of cartilage repair. I thank him for placing trust in me, and letting me grow as an individual in science and in life. I feel extremely fortunate to have him as a guide to tide over the most challenging and most fruitful turning point of my professional life. Over a period of time, his ideas and thoughts have moulded me into a better researcher and enabled me to develop a genuine appreciation for good science. His vision and structured thinking have made tremendous contribution in driving my research and writing. I am thankful for several opportunities to attend and present at international conferences which added to my growth as a professional. During the course of my study, I encountered some difficult times and I am grateful for his immense support, understanding and patience. I am certain that his wise words will continue to guide me in the future.

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RÉSUMÉ

Le cartilage articulaire hyalin recouvre l'extrémité des os longs. Sa capacité de guérison intrinsèque est faible puisqu'il n'est pas vascularisé. Suite à une blessure, la guérison n'a généralement pas lieu, ou demeure de faible amplitude. Une dégénérescence des lésions peut également survenir suite au relargage de cytokines et autres facteurs inflammatoires. Un déséquilibre entre les activités anaboliques et cataboliques peut éventuellement induire l'érosion du cartilage, l'exposition de l'os sous-jacent et l'ostéoarthrite.

Les techniques de stimulation de la moelle osseuse sont une façon de stimuler la guérison du cartilage en perçant l'os situé sous les lésions du cartilage. Les canaux percés permettent aux cellules progénitrices qui se trouvent dans la moelle osseuse (bone marrow progenitor cells en anglais) de migrer vers les lésions. Ces cellules progénitrices se différencient et synthétisent un tissue de granulation qui sera remodelé en tissue de guérison. Les techniques de stimulation de la moelle osseuse sont beaucoup utilisées en clinique puisqu'elles sont simples et peu coûteuses à effectuer, que les patients se rétablissent rapidement après la chirurgie et qu'elles sont efficaces pour diminuer la douleur. Cependant, les bienfaits ressentis ne sont pas durables puisque le tissu de guérison est un tissue fibrocartilagineux qui ne possède pas les propriétés biologiques et mécaniques du cartilage hyalin. Les inconvénients les plus communs observés au cours d'études pré-cliniques et cliniques portant sur les techniques de stimulation de la moelle osseuse sont une guérison incomplète, une grande variabilité inter-individu, et des résultats insatisfaisants, surtout chez les individus plus âgés. Il a déjà été démontré dans des études pré-cliniques que l'emplacement de la lésion ainsi que l'âge de l'animal sont deux paramètres qui affectent grandement la guérison. Plus spécifiquement, chez le lapin, les lésions situées sur la trochlée guérissent mieux que les lésions situées sur le condyle fémoral médial, ce qui suggère que les cellules progénitrices de la moelle osseuse présentes aux deux emplacements ne possèderaient pas les mêmes propriétés biologiques intrinsèques.

L'objectif de la première étude de cette thèse était de déterminer l'effet de l'emplacement et de l'âge de l'animal sur les propriétés biologiques intrinsèques des cellules progénitrices de la moelle osseuse. Pour ce faire, les propriétés biologiques des cellules isolées à partir de la trochlée et des condyles chez des lapins jeunes et âgés ont été caractérisées. Ensuite, puisque de

précédentes études chez le lapin ont démontré qu'un perçage profond à 6 mm favorise la guérison du cartilage dans la trochlée en comparaison à un perçage peu profond, les propriétés biologiques de cellules progénitrices isolées à partir de la moelle métaphysaire (région profonde) et de la moelle épiphysaire (région peu profondes) ont été caractérisées. Deux méthodes d'isolation des cellules progénitrices ont été utilisées, soit la digestion enzymatique à la collagénase et la culture d'explants osseux. Pour chaque population de cellules progénitrices, le rendement cellulaire, le potentiel clonogénique et l'expression de différents marqueurs ont été déterminés *in vitro*. Le potentiel chondrogénique a, quant à lui, été déterminé en stimulant les cellules progénitrices avec un milieu de culture chondrogénique contenant de l'ascorbate, ITS et TGF-βIII. Enfin, le potentiel ostéogénique a été déterminé en stimulant les cellules progénitrices avec un milieu de culture ostéogénique contenant du dexamethasone, de la β-glycerophosphate et de l'ascorbate. Une comparaison entre les propriétés biologiques obtenues pour les cellules progénitrices provenant des condyles, de la trochlée profonde, ou de la trochlée peu profonde, pour les lapins jeune ou âgés et pour les cellules isolées par digestion enzymatique ou par culture d'explants a ensuite été effectuée. Selon les critères établis par l'International Society for Cell Therapy, les cellules progénitrices isolées lors de l'étude pouvaient être décrites comme une population de cellules souches mésenchymateuses. Les condyles avaient un faible rendement cellulaire en comparaison à la trochlée et les cellules progénitrices isolées à partir des condyles avaient également de faibles potentiels clonogénique et chondrogénique. En revanche, les cellules progénitrices isolées à partir de la trochlée possédaient un haut rendement cellulaire, des potentiels clonogénique et chondrogénique élevés et exprimaient beaucoup de glycosaminoglycanes et de collagène type II, deux molécules présentes dans le cartilage articulaire hyalin. Le site d'isolation ou l'emplacement était donc un facteur déterminant pour les propriétés biologiques des cellules progénitrices, ce qui suggère que le potentiel de guérison élevé observé dans la trochlée du lapin serait dû à la présence d'une population nombreuse de cellules progénitrices ayant un fort potentiel chondrogénique. L'âge des lapins s'est aussi avéré être un facteur important puisque le rendement cellulaire, l'expression de marqueurs pour cellules souches et la différenciation chondrogénique et ostéogénique étaient meilleurs chez les lapins jeunes que chez les lapins âgés. Enfin, il n'y avait pas de différence entre les cellules isolées par digestion enzymatique ou par culture d'explants.

L'objectif de la deuxième étude de cette thèse était de déterminer s'il existe une corrélation entre les propriétés biologiques intrinsèques des cellules progénitrices de la moelle osseuse et la capacité de guérison du cartilage sus-jacent. Pour ce faire, un modèle bilatéral a été effectué chez 8 lapins. Pour chaque lapin, des lésions cartilagineuses ont été créées sur un genou à deux emplacements (la trochlée et le condyle fémoral médial) puis traitées par microperçage, tandis que l'autre genou demeurait intact. Après 3 semaines de guérison, les lapins ont été sacrifiés et des sections histologiques ont été récoltées du genou traité par microperçage afin d'évaluer la guérison. Parallèlement, les cellules progénitrices du genou intact ont été récoltées et leurs propriétés biologiques caractérisées tel que décrit ci-haut. Chez tous les lapins, la guérison induite par stimulation de la moelle osseuse était compromise dans le condyle fémoral médial, où très peu de tissu de guérison était présent après 3 semaines. En revanche, une guérison modérée à excellente était apparente dans la trochlée de 6 lapins sur 8, alors que 2 lapins ont été classifiés comme faisant partie d'un groupe ayant un plus faible potentiel de guérison. Tout comme pour la première étude, les cellules progénitrices de la trochlée avaient un meilleur rendement cellulaire et de forts potentiels clonogénique et chondrogénique que les cellules des condyles. Une variabilité inter-individu était également présente dans le cas des propriétés biologiques des cellules progénitrices. Les tissus de guérison de la trochlée ainsi que la matrice extracellulaire synthétisée par les cellules progénitrices isolées à partir de la trochlée contenaient une grande quantité de glycosaminoglycanes et de collagène type II. Des analyses statistiques ont permis de déterminer qu'il existait de fortes corrélations positives entre les propriétés intrinsèques des cellules progénitrices de la moelle osseuse et la capacité de guérison du cartilage sus-jacent. Les propriétés biologiques ayant le plus d'impact sur la guérison ont été identifiées.

L'objectif de la troisième étude de cette thèse était de développer un modèle animal représentatif de la situation clinique, et d'y tester une nouvelle méthode de guérison. En clinique, les lésions du cartilage sont rarement détectées rapidement, ce qui complique le traitement. Une grande variabilité de la réponse et une guérison compromise sont également observées chez les patients plus âgées souffrant de lésions dégénératives chroniques. La rétraction du caillot sanguin qui remplit les lésions cartilagineuses suite au microperçage et sa perte subséquente sont une raison qui pourrait expliquer les résultats insatisfaisants observés en clinique. Il a été démontré que le chitosane permet d'inhiber la rétraction du caillot sanguin et, par le fait même, qu'il promeut la guérison du cartilage en ayant des effets sur le recrutement cellulaire, la vascularisation and le

remodelage osseux. Le plasma riche en plaquettes (PRP) contient une concentration élevée de facteurs de croissance capables de stimuler le recrutement cellulaire et la différenciation chondrogénique. Il a été démontré que le chitosane favorise la stabilité du PRP et le relargage de facteurs de croissance. Un modèle de lésions cartilagineuses chroniques a d'abord été développé chez le lapin. Deux chirurgies était nécessaire pour effectuer ce modèle. Au cours de la première chirurgie, des lésions cartilagineuses bilatérales ont été induites dans la trochlée de 8 lapins et laissées sans traitement pendant 4 semaines afin qu'elles dégénèrent à un stade chronique. Au cours de la deuxième chirurgie, ces lésions cartilagineuses ont été traitées par microperçage et par l'implantation de chitosane lyophilisé solubilisé dans du PRP autologue, ou par injection de PRP seul, comme contrôle. Le tissu de guérison a été récolté après 2 mois et des sections histologiques ont permis d'évaluer la quantité et la qualité du tissu de guérison. La guérison osseuse ainsi que le remodelage osseux ont également été caractérisées par analyse micro-CT. Les implants chitosane-PRP favorisaient la guérison des lésions cartilagineuses chroniques comparées au PRP seul. La composition biochimique de la matrice cartilagineuse ressemblait plus à du cartilage hyalin en présence des implants chitosane-PRP. Finalement, l'analyse micro-CT démontrait que les implants chitosane-PRP favorisent le remodelage osseux mais que le phénomène est toujours en cours après 2 mois de guérison.

En résumé, le contenu de cette thèse nous a permis d'identifier certaines variables liées à l'emplacement, au donneur et à l'âge qui expliqueraient partiellement la variabilité inter-individu et les résultats décevants parfois obtenus suite à une stimulation de la moelle osseuse. Le modèle de lésions chroniques décrit dans cette thèse contribuera grandement au développement de nouvelles techniques et de nouveaux produits pour favoriser la guérison du cartilage articulaire. Enfin, les implants chitosane-PRP étudiés au cours de cette thèse constituent une approche prometteuse qui pourrait éventuellement servir à soulager des patients souffrant de lésions chroniques ou dégénératives.

ABSTRACT

Articular cartilage is a thin layer of hyaline cartilaginous tissue covering the ends of long bones. Due to its avascular nature, it possesses very limited regeneration potential. In the event of an injury, the repair is either very limited or not initiated at all. As a result, cartilage lesions degenerate extensively under the influence of damaging cytokines and proinflammatory factors released. An imbalance in anabolic and catabolic activities continues to erode the cartilaginous surface exposing subchondral bone leading to osteoarthritis.

Bone marrow stimulation (BMS) initiates repair by fracturing or drilling into subchondral bone. The channels created provide access to the underlying subchondral progenitor cells that are recruited into the defect. Granulation tissue thus formed undergoes a complex cascade of events to generate a repair tissue. BMS is considered a gold standard of cartilage repair strategies since it is easy, relatively less invasive with swift recovery, more economical and appreciable short-mid term relief. However, long term outcome is generally discouraging due to poor durability of repair tissue. The quality of repair tissue is generally fibrocartilagenous with inferior mechanical and biological characteristics compared to hyaline cartilage. The most common drawbacks of the procedure include incomplete regeneration, high inter-individual variability and poor repair outcome especially in older individuals in preclinical as well as clinical studies. Earlier studies have shown that defect location and animal age affect the cartilage repair outcome observed *in vivo*, suggesting a strong influence of biological characteristics of progenitor cells (Bone Marrow stem cells; BMSCs or mesenchymal stem cells; MSCs) present in subchondral bone. Specifically, rabbit trochlea showed a superior repair and chondrogenic potential compared to the medial femoral condyle. Inspired by these observations, we carried out a study for comprehensive analysis of progenitor cells present in the subchondral bone of condyle and trochlea in young and old rabbits in order to determine the influence of location and age on the properties of progenitor cells. Earlier we had found that drilling to 6 mm improves the repair outcome in rabbit trochlea possibly by providing access to metaphyseal marrow and thus higher progenitor cell population. As a result, MSCs were isolated from epiphyseal (trochlea upper) and metaphyseal (trochlea lower) regions of the trochlea. Collagenase-derived MSCs originated from collagenase digest of bone chips while the digested explants were subsequently cultured to obtain explant-derived MSCs via cell outgrowth. *In vitro* characterization assays were used to determine the cell yield, clonogenic potential and surface marker expression profile. Chondrogenic differentiation was

stimulated using pellets of condylar and trochlear MSCs in the presence of chondrogenic media, rich in chondrogenic stimulating factors such as ascorbate, insulin-transferrin-selenium (ITS) and TGF-βIII. Finally, MSCs were differentiated into osteogenic lineage in presence of osteogenic media containing osteogenic stimulants such as dexamethasone, β-glycerophosphate and ascorbate. The aforementioned MSC characteristics were compared for trochlea vs. condyle, old vs. young and collagenase- vs. explant-derived cultures. MSCs isolated from distal femur epiphyseal bone had stem cell characteristics and fulfilled the stem cell criteria outlined by International Society for Cell Therapy. Location was an important factor influencing the properties of MSCs indicated by low cell yield, clonogenic potential and inferior chondrogenic potential of condylar cells. Results showed that rabbit trochlear vs. condylar subchondral bone yielded a greater number of progenitors with superior cartilaginous matrix expression under chondrogenic conditions suggesting higher intrinsic capacity for cartilage repair compared to condylar subchondral bone. Trochlear MSCs displayed superior chondrogenic differentiation evidenced by higher glycosaminoglycan (GAG) and collagen type II deposition. Age of the donor was an additional factor with significant bearing on the inherent properties of MSCs since growth rate, cell yield, expression of stem cell markers and osteogenic differentiation were found to be significantly superior in younger animals. As expected, no significant difference was observed between collagenase- and explant-derived cultures.

Continuing our exploration of the mechanisms underlying variability in BMS procedure, we carried out the next study to verify if the cartilage repair outcome in condyle and trochlea correlated with the pre-existing properties of progenitor cells present in these two sites. A bilateral rabbit model was used to test this hypothesis. For each rabbit, full thickness acute defects were created in one knee and treated by BMS by microdrilling, while the second knee was left intact. Animals were sacrificed 3 weeks later and transverse sections of repair tissue from the treated knee were used to analyze the quality and quantity of repair macroscopically and by safranin- O (Saf-O) and immunostaining. At the time of animal sacrifice, distal femurs were separated and MSCs were isolated from condylar and trochlear regions of the intact contralateral knee for biological characterization of cell yield, clonogenic potential, cell surface marker profile and differentiation potency. BMS induced inferior cartilage repair in condyles of all donors studied further underlining the implication of location in influencing repair outcome. Macroscopic examination revealed extremely poor fill in condylar defects. In contrast, moderate

to excellent fill was observed in six out of eight trochlear defects. This led us to identify a donor-dependent variability in repair outcome and the donors were classified as good trochlear responders and poor trochlear responders. In another observation, trochlear MSCs were characterized by increased cell yield, higher clonogenic potential and superior chondrogenic potential. Histopathological analysis indicated better matrix composition, rich in GAG and collagen type II in trochlea both *in vivo* and *in vitro*. Donor related variability in repair outcome was also observed *in vitro* through biological characterization of MSCs. Statistical analysis provided substantial evidence to identify strong, positive location- and donor-dependent correlations between inherent properties of MSCs and cartilage repair outcome in condyle and trochlea and factors with maximum influence on the repair outcome were identified.

By this stage, it became imperative to seek after a clinically relevant application of our understanding based on current and past findings. Cartilage lesions are rarely detected in early stages, unfortunately reducing the efficacy of BMS procedure. Unpredictable, highly variable repair of poor quality is observed in degenerative defects, especially in older patients. Platelet-mediated retraction of the blood clot that fills cartilage lesions following BMS is believed to be one underlying cause of inferior quality and durability of repair tissue. Chitosan inhibits this blood clot shrinkage and leads to the formation of a voluminous, adherent and stable clot and thereby enhances cartilage repair by promoting cell recruitment, transient vascularization and subchondral bone remodeling. Platelet-rich-plasma (PRP) contains a 2-10 fold concentration of growth factors and cytokines and has been shown to improve recruitment and chondrogenic potential of subchondral MSCs. Chitosan increases the stability of PRP and promotes the release of platelet-derived growth factors in a more sustained manner. Using a more clinically relevant chronic defect model, we tested the efficacy of implants composed of freeze-dried chitosan solubilized in PRP in improving marrow stimulated cartilage repair outcome compared to BMS augmented with just PRP. Two surgeries were performed in skeletally mature rabbits to create a bilateral trochlear defect model. Defects created in the first surgery were allowed to degenerate over 4 weeks and repaired in a second surgery by BMS. Treatments included BMS augmented with chitosan/PRP implants in one knee and BMS with PRP in the other knee. Repair tissue was harvested 2 months later and transverse sections were used to characterize the quality and quantity of repair by assessing the GAG and coll-II deposition. Subchondral bone repair was analysed by micro-CT. Augmentation of BMS with freeze-dried chitosan/PRP implants in

chronic defects improved cartilage repair compared to PRP by increasing quantity and quality of repair tissue and promoting subchondral bone repair. Matrix composition was improved in presence of chitosan/PRP indicated by increased GAG and collagen deposition. MicroCT analysis showed further evidence of increased, albeit incomplete, subchondral bone remodeling induced by chitosan/PRP.

In summary, these studies led us to recognize significant donor-, age- and location-dependent variability which might help in understanding the mechanism underneath the unpredictability and inter-individual variability observed with BMS. Needless to say, these observations would be extremely valuable in improving the consistency, durability and quality of BMS repair procedure. Moreover, the innovative chronic model studied here is expected to make significant contributions in progress of cartilage repair field. Finally, chitosan/PRP implants investigated in this research are a promising approach to treat chronic cartilage lesions and could eventually provide relief to osteoarthritic patients with challenging, degenerative defects.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
κ	Kappa
Ac	Acetyl
AC	Articular cartilage
ACI	Autologous Chondrocyte Implantation
ADSC	Adipose derived stem cell
AGE	Advanced glycation end product
APase	Alkaline Phosphatase
ARS	Alizarin Red S
BM	Bone Marrow
BMP	Bone Morphogenetic Proteins
BMPC	Bone marrow progenitor cell
BMSC	Bone marrow stem cell
BMS	Bone Marrow stimulation
BS	Bone surface area
BSA	Bovine Serum Albumin
BV	Bone volume
$CaCl_2$	Calcium chloride
cAMP	cyclic AMP
CC	Calcified cartilage
CCM	Control media
CD	Cluster of Differentiation

Cfkh1	Chicken Forkhead 1
CFUf	Colony Forming Unit-fibroblastic
Col-I	Type I collagen
Col-II	Type II collagen
Col-IV	Type IV collagen
Col-X	Type X collagen
Col-XI	Type XI collagen
COMP	Cartilage oligomatrix protein
CPC	Cetylpyridinium Chloride
CS	Chitosan
CT	Computed tomography
DB	Bone debridement
DDA	Degree of deacetylation
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DRL	Drill
ECM	Extracellular matrix
EDTA	Ethylene Diamine Tetraacetate
EGF	Epithelial Growth Factor
EO	Endochondral ossification
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FG	Fast Green
GAGs	Glycosaminoglycans

GDF	Growth and differentiation factor
GF	Growth factor
GLM	General Linear Model
GT	Granulation tissue
βGP	β-Glycerophosphate
HA	Hyaluronic acid
HBSS	Hank's Buffered Salt Solution
HCl	Hydrochloric Acid
H&E	Hematoxylin and Eosin
HLA	Human Leukocyte Antigen
Hu	Human
HSC	Hematopoetic stem cell
HT	Chondrocyte Hypertrophy
IGF	Insulin Growth Factor
ICRS	International Cartilage Repair Society
ISCT	International Society of Cell Therapy
IHH	Indian Hedgehog
IL	Interleukin
ISCT	International Society of Cell Therapy
ITS	Insulin Transferrin Selenium
JNK	Jun amino-terminal kinases
kDa	kilodalton
kV	kilovolt
LIF	Leukemia inhibitory factor

µ	Micro
M	Molar
MAPK	Mitogen Activated Protein Kinase
MEM	Minimum Essential Medium
MfH1	Mesenchymal Fork head-1
MFx	Microfracture
MFC	Medial femoral condyle
miR	micro RNA
MMP	Matrix Metalloproteinase
MPC	Mesenchymal Progenitor Cell
MSC	Mesenchymal stem cell
MSK	Mitogen- and stress-activated protein kinase
NBF	Normal Buffered Formalin
OA	Osteoarthritis
OSM	Osteogenic media
P0	Passage 0
PCAF	p300/CBP-associated factor
PCL	Poly caprolactone
PDGF	Platelet Derived Growth Factor
PGE2	Prostaglandin E2
PLLA	Poly-L-Lactic acid
PMN	Polymononuclear cells
PPAR	Peroxisome proliferator-activated receptor
PRP	Platelet-Rich Plasma

P-S	Penicillin Streptomycin
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone related Peptide
PTOA	Post traumatic osteorthritis
r	Pearson Correlation coefficient
r^2	Square of Pearson correlation coefficient
Rb	Rabbit
RNA	Ribonucleic acid
RT	Repair tissue
Runx	Runt-related transcription factor
Saf-O	Safranin-O
SDF	Stromal-derived factor
SR	Spontaneous repair
TE	Tissue Enginneering
TGF	Transforming Growth Factor
TIMP	Tissue inhibitors of metalloproteinase
TNF	Tumour Necrosis Factor
TV	Total volume
VEGF	Vascular Endothelial Growth Factor
VCAM	Vascular cell adhesion molecule
VI	Vascular invasion
Wnt	Wingless int
w/v	Weight by volume

CHAPTER 1 INTRODUCTION

1.1 Introduction

The ends of long bones such as femurs are covered by a thin layer of cartilaginous tissue known as articular cartilage [1]. It is a highly specialized skeletal tissue and derives its unique biomechanical and physiological properties from an intact matrix comprised of proteoglycans and collagen type II [2]. Since this tissue lacks blood, nervous and lymphatic supply, in the event of an injury, the regeneration process is either not initiated or is insufficient to restore healthy tissue. Chondrocytes, the primary cell type of cartilage- are embedded in copious amount of ECM and are unable to migrate to populate the region of defect. Negligible blood supply further aggravates the problem since the stimulatory signals required to drive a repair process fail to propagate [3]. Full thickness lesions which reach sub-chondral bone undergo a limited repair process due to the access to the progenitor cells in the bone marrow stroma, though the quality of the repair tissue is mainly fibrocartilagenous with inferior mechanical and physiological properties On the other hand, partial thickness defects are not repaired at all [4, 5].

The knee joint is the most commonly affected joint and accounts for 75% of all lesions observed in articular cartilage. In an early study involving over 31,000 patients, it was found that while 63% knees had chondral lesions, 20% suffered from full thickness lesions [6]. It is expected that 60% of the patients who develop cartilage lesions will have osteoarthritis (OA) within 20 years of generating an articular cartilage defect [6]. Cartilage loss generally occurs due to age-related wear and tear and the likelihood of its degradation increases with increasing age. In a population with increasing life expectancy, up to 40% of population above 70 could be expected to be affected by OA leading to a burden of 5-20 billion dollars on the Canadian government [7]. However, trauma related osteoarthritis is not uncommon and although focal at onset, may progress into OA if not addressed swiftly. Cartilage degeneration is multifactorial and involves femoral, patellar and synovial compartments. Catabolic activities mediated by pro-inflammatory cytokines such as IL-1 and TNF-α are not balanced by anabolic growth factors like IGF-1 and TGF-β and anti-inflammatory cytokines like IL-4 and IL-10 [8, 9]. The disrupted equilibrium leads to degeneration of matrix exposing the subchondral bone and causing patient discomfort [1-5].

Bone marrow stimulation (BMS) initiates cartilage repair by fracturing or drilling into subchondral bone at the base of a debrided cartilage defect, typically leading to the formation of a fibrocartilagenous repair tissue providing varied levels of clinical relie-f. It is relatively easy, economical, associated with swift recovery and provides appreciable short to mid term relief. Due to advantages offered by the procedure, BMS is adopted as first line of treatment globally. However, repair outcomes are affected by defect location and age, suggesting a strong influence of the structural and biological factors in the knee joint. These differences may include anatomical differences in the structure of knee as well as biological properties of mesenchymal progenitor cells in the subchondral bone. It is well known that human trochlear defects are rarer but harder to treat compared to condyles [10], while animal models present varying scenarios. In an earlier study, rabbit trochlea showed a superior repair and chondrogenic potential compared to medial femoral condyle [11, 12]. On the other hand, quality of repair was better in condyle than trochlea in a sheep model [13]. Taken together, these studies emphasize the need to address the inconsistent repair outcomes arising from location-dependent factors. Unfortunately, BMS procedures are also severely affected by increasing age leading to incomplete regeneration, high inter-individual variability and poorer outcome in older individuals. Therefore, identification of mechanism for reduced potential of progenitor cells in cartilage repair is required in order to understand fundamental causes of poor repair in older individuals. The exact mechanism of donor-, location- and age-dependent variability still eludes us since our knowledge about underlying factors such as bone structure and mechanics, load-bearing conditions and the role of subchondral bone progenitor cells is still extremely limited. A better understanding of the underlying causes and mechanism of variability in the marrow stimulated repair outcomes will enable us to make the approach more predictable, clinically efficient and personalized to patient's needs. In addition, it will be pave the way to develop strategies to enhance the quality of repair, especially in hard to treat cases.

BMS provides access to the underlying bone marrow stroma rich in bone marrow progenitor cells (BMPCs)/bone marrow stem cells (BMSCs)/mesenchymal stem cells (MSCs) by creating channels in the subchondral bone [14]. BMSCs are characterized by a unique potential of self renewal and ability to differentiate into cells of multiple lineages [15]. When recruited to the site of defect, they differentiate into chondrogenic phenotype leading to the formation of a repair tissue with varying amounts of hyaline and fibrous cartilage [16]. Since BMS mediated repair of

soft and hard tissues relies on recruitment of underlying BMSCs, it is logical to explore the properties of these progenitors in order to provide important mechanistic insights into variability present in marrow stimulated repair outcomes. It is reasonable to assume that differences in number, stemness and differentiation potential of BMSCs will influence the chondrogenesis *in vitro* and cartilage repair *in vivo*. Biological characterization can be carried out *in vitro* by means of several assays including determination of cell yield, CFU-f, expression of cell surface markers and multilineage differentiation assays. In a previously published study, our group demonstrated superior repair when trochlea was drilled to provide access to metaphyseal marrow [17]. Due to likely differences in the progenitor cell population in the epiphyseal and metaphyseal regions of bone, and their subsequent influence on repair response, the cells derived from these locations need to be characterized individually. Cells participating in BMS include cortico-spongious bone and marrow spaces and therefore characterization of cells originating from both these sources is warranted.

Though repair response induced by BMS along with characterization of BMSCs has been investigated in animal models before [18-21], a study designed to correlate the repair response in different anatomic locations with biological properties of BMSCs is lacking. Needless to say, inherent differences in the properties of BMSCs can be expected to influence cartilage repair outcomes in a profound manner. A statistically sound study designed to investigate the influence of location- and donor-dependent factors on cartilage repair outcomes is more likely to elucidate the underlying mechanism of inter-individual variability observed in cartilage repair. Inferences from such an analysis will probably aid clinicians in developing repair strategies on a more patient-relevant basis.

Another reason for poor quality of BMS repair is the compromised residency and stability of marrow-derived blood clot that fills cartilage lesions following bone fracturing [13, 18, 19]. Due to platelet-mediated clot retraction and serum exudation, the clot can lose more than half of its original volume. A retracted clot will eventually lead to early detachment of repair tissue from bone bed undermining the efficacy of this procedure. In contrast, a voluminous, physically adherent and stable clot is generated when chitosan - a biocompatible and biodegradable polymer comprised of glucosamine and N-acetyl glucosamine residues - is mixed with autologous blood prior to clotting and leads to an improved osteochondral repair [13]. Platelet-rich-plasma (PRP)

containing concentrated platelets is a rich source of growth factors, cytokines and chemokines [22-24] and has been shown to provide adequate stimulation for recruitment and chondrogenic differentiation of BMSCs [25, 26]. However, PRP clots are even more prone to retraction which might lead to insufficient chondrogenesis and hyaline cartilage restoration as reported by some authors [27]. A combination of freeze-dried chitosan and PRP would increase the stability and bioactivity of PRP likely improving cartilage repair outcomes in challenging cases. Liquid chitosan in aqueous solution suffers from limited shelf life due to poor stability during storage [28]. Therefore, freeze-dried chitosan offers the advantages of improved stability, longer shelf life and easier sterilization.

The principle of augmented marrow-stimulation relies on the enhanced recruitment of mesenchymal progenitor cells to the lesion accompanied by increased stability of growth factors and BMSCs in the defect milieu. It is important to bear in mind that cartilage repair does not occur in isolation and inflammatory mediators in defect milieu are likely to complicate the healing process. Therefore, it becomes imperative to examine cartilage repair strategies in a model which is a more realistic depiction of clinical situation. Interplay of catabolic factors including damaging cytokines and pro-inflammatory factors are more likely to be represented in a model with degenerative, chronic lesions [29, 30]. To ensure successful clinical translation, it is critical to explore the potential of chitosan and PRP in improving the repair outcome in chronic lesions which are harder to treat compared to acute lesions.

The objective of the study presented here is to make significant contributions to enhance the existing understanding of variability and unpredictability in marrow stimulated repair outcomes. The research aims to address the gap in our current comprehension of the underlying mechanisms of donor-, age- and location-dependent inconsistency observed with BMS, likely arising due to inherent properties of mesenchymal progenitor cells. Correlation of intrinsic biological properties of progenitor cells and cartilage repair outcome will eventually enable clinicians to perform BMS in a more patient-specific basis where intrinsic repair capacity is highly variable and unpredictable. Finally, conclusions from analysis of BMS augmented with chitosan/PRP implants in a chronic lesion will likely offer a promising strategy specifically designed to enhance the cartilage repair outcome in demanding cases.

1.2 Research Hypotheses and Objectives

1.2.1 General objective

The research presented here was carried out with an objective to explore the underlying mechanism of variability in marrow stimulated repair outcome by identifying location-, age- and donor-dependent factors influencing the inherent properties of subchondral bone progenitor cells (or mesenchymal stem cells, MSCs). Furthermore, strategies to improve cartilage repair outcome mediated by augmented BMS techniques were investigated in animal studies designed for a more accurate representation of the chronic lesions present in the clinical situation.

1.2.2 Study 1

The aim of this study was to carry out a comprehensive analysis of the progenitor cells present in the subchondral bone of rabbit condyle and trochlea, taking into account location and age revealing a likely mechanism for variability in marrow stimulated cartilage repair seen *in vivo*.

1.2.2.1 Hypothesis1

We hypothesized that the inherent biological properties of bone marrow progenitor cells (BMPCs; or mesenchymal stem cells, MSCs) including number, clonogenicity, surface marker profile and multilineage differentiation potential will be influenced by location and age while origin of the cells will have no significant bearing on these properties. Specific hypotheses tested through this study included the following:

- *In-vitro* biological properties including P0 cell yield, clonogenic potential, expression of cell surface markers, chondrogenic and osteogenic differentiation potential will be superior for BMPCs/MSCs isolated from rabbit trochlea compared to condyles.
- Cell yield, stemness markers, clonogenic, chondrogenic and osteogenic potential of BMPCs/MSCs will decrease with increasing age.
- The aforementioned *in vitro* biological properties of BMPCs/MSCs will not be affected by the method of isolation and will be similar for collagenase- and explant-derived BMPCs/MSCs.

1.2.2.2 Objective 1

Isolate progenitor cells (BMPCs/MSCs) from condyles and trochlea of young and old rabbits by collagenase digestion of marrow and explant culture followed by comprehensive analysis of their biological properties including- a). Cell yield, b). CFU-f, c). Expression of surface markers characteristic of bone marrow progenitor cells and d). Multilineage differentiation potential - in order to identify the factors influencing inherent properties of progenitor cells and likely to impact marrow stimulated repair outcome.

1.2.3 Study 2

The aim of this study was to determine the location- and donor-dependent correlation between marrow-stimulated cartilage repair outcome and inherent biological properties of BMSCs in order to identify the association between cellular behaviour of progenitor cells and variability in marrow stimulated repair response.

1.2.3.1 Hypothesis 2

Cartilage repair outcome in trochlea will be superior to condyles in an acute rabbit defect model and correlate to the pre-existing *in vitro* biological properties of the BMSCs at the two sites in the contralateral knee. Specific hypotheses tested were:

- The quality as well as quantity of condylar repair tissue will be inferior to trochlear repair tissue indicated by lower volume of repair tissue and reduced GAG and collagen type II deposition.
- Inferior repair outcome in condylar defect will correlate positively with reduced cell yield, lower clonogenic and inferior multilineage differentiation potential of condyles determined *in vitro*.

1.2.3.2 Objective 2

Develop a bilateral model for repair of acute full thickness defect in condyle and trochlea in one knee and biological characterization of cells isolated from these 2 sites in the contralateral knee. Using appropriate statistical model, correlate the biological properties of BMSCs isolated from condyles and trochlea with early (3 weeks) repair outcome assessment parameters.

1.2.4 Study 3

This study was carried with an aim to develop a strategy to enhance cartilage repair response, especially in challenging conditions of extensive damage and degeneration. Role of PRP and Chitosan/PRP implants in augmentation of marrow stimulated cartilage repair outcome was investigated in a chronic defect model using quality and quantity of repair tissue as outcome assessment parameters.

1.2.4.1 Hypothesis 3

Repair outcome will be better with BMS augmented with freeze-dried chitosan/PRP implants compared to BMS augmented with recalcified PRP in an adult chronic trochlear defect model.

1.2.4.2 Objective 3

Optimize rabbit chronic defect model and apply it to analyze the outcome of cartilage repair as a result of BMS augmented with freeze-dried chitosan/PRP implants. Use macroscopic regeneration and matrix composition of repair tissue along with subchondral bone regeneration to assess and compare the repair outcome.

CHAPTER 2 LITERATURE REVIEW

2.1 Articular Cartilage

2.1.1 Overview

Articular cartilage is a highly specialized tissue, avascular, aneural and alymphatic and at maturity of very low metabolic activity [1]. The main functions of articular cartilage include: a. Act as a shock absorber and withstand the compression, tension and shearing forces characteristic of a highly active joint such as knee, b. Distribute load, c. Provide a smooth, frictionless articulating surface and prevent damage to the underlying bone. Articular cartilage lacks any blood or lymphatic supply and derives its nutrition through synovial fluid present in the synovial joint as well as the underlying bone [31]. The exchange of the metabolites, gases and waste products also takes place through synovial fluid.

2.1.2 Composition of articular cartilage

2.1.2.1 Zonal organization

The medial condyle in young adults is ~2.4 mm thick lying on top of a ~130 µm thick calcified cartilage layer followed by a subchondral bone plate ~190 µm thick [32]. The biochemical and biophysical properties of cartilage are well adapted to bear repetitive mechanical forces which may even reach up to 65 times body weight. The articular cartilage is composed of a network of collagen fibres and a proteoglycan matrix within which reside cartilage cells. Healthy cartilage is organised into four zones comprising a superficial tangential zone characterized by flattened chondrocytes, a middle zone which is comprised of rounded chondrocytes, followed by a deep zone where the chondrocytes are spherical and columnar. Calcified cartilage comprised of very small chondrocytes forms the fourth inner most zone which is the region of extensive shear stress [2].

2.1.2.2 Cells

The articular cartilage is unique in being composed of mainly just one type of cell, i.e. chondrocytes which secrete the ECM that provides the tissue's structure and contributes to the

functional, mainly mechanical role of cartilage. Chondrocytes represents only about 1% of the volume of articular cartilage. Chondrocytes also modify matrix properties in response to loading due to various mechanotransduction mechanisms [33]. Chondrocytes are mesenchymal in origin and differentiate from mesenchymal stem cells in bone marrow. During development the BMSCs differentiate into chondrocytes with those at the periphery of the bone and cartilage developing into hypertrophic chondrocytes and bringing about calcification and endochondral ossification. [1, 33].

2.1.2.3 Extracellular Matrix

The cartilage matrix is particularly rich in collagens and proteoglycans. The tissue possesses high tensile strength owing to the presence of collagen while the proteoglycan matrix which can hold up to 75-80% water contributes to the tissue's capability of resisting compressive forces, one of the most important functions of articular cartilage [34]. Collagens form up to 75% of dry tissue weight. 90-95% of matrix collagen is type II, which due to associated carbohydrates; promotes interaction with water that is a critical requirement for the functional property of cartilage. Type X is mainly present in calcified cartilage and is secreted by hypertrophic chondrocytes [34]. 20-25% of cartilage matrix is composed of proteoglycans such as HA, chondroitin sulphate, keratin sulphate, heparin sulphate [1, 33]. The proteoglycans are composed of 95% polysaccharides and 5% proteins. The glycosaminoglycans associated with proteins play a major role in interaction with water. The interaction of these GAGs with water lends the property of flexibility to cartilage in addition to displaying excellent mechanical strength and shock resistance properties.

2.1.3 Development of articular cartilage

The embryonic development of cartilage begins from mesenchyme at the 5^{th} week of gestational age by aggregation of mesenchymal cells to form blastema. These cells express cartilage matrix and develop into chondroblasts. The increasing amount of matrix pushes the cells further away from each other. The cells become encased in the dense and copious amount of matrix and become chondrocytes. Meanwhile, perichondrium is formed by the mesenchymal tissue surrounding the blastema [35]. In their study using New Zealand White rabbit model, Hunziker *et al* revealed important insights into post natal development and growth of bone and articular cartilage [36]. A temporal trend was clear in the structural maturation of the articular cartilage

layer. The growth activity of the articular cartilage layer was highest in the period following birth and declined quickly between the first and third months. The growth ceased when the animal achieved the puberty between the third and fourth months. The growth activity in the true metaphyseal growth plate continued until sexual maturity-8 months in rabbit indicating independent regulation of the surface growth plate and metaphyseal growth plate. They found that the postnatal reorganization of articular cartilage from an immature isotropic to a mature anisotropic structure results from resorption and neoformation of all zones except the most superficial zone and not by a process of internal remodelling where the superficial zone was comprised of slowly dividing stem cells with bidirectional mitotic activity. The stem cells in this zone drive the lateral expansion of the AC layer in addition to replenishing the stem cell pool. In addition, the zone is also responsible for the postnatal growth phase of the articular cartilage layer by providing rapidly dividing, transit-amplifying daughter-cell pool that feeds the transitional and upper radial zones. Therefore, mammalian articular cartilage not only as an articulating layer but also as a surface growth plate in the post natal development [37].

2.1.4 Articular cartilage defects

In a review with 993 arthroscopies, 66% of knees contained cartilage pathologies as measured by ICRS scoring system [6]. In the US, approx. 900,000 individuals are affected by chondral lesions per annum as a result of which more than 200,000 surgical procedures are needed to treat grade III and IV defects [38]. In another study with 31,000 arthroscopic procedures, an alarming rate of 63% incidence of cartilage defects was noted with 41% grade III and 19% grade IV incidence [39]. In 1,000 knee arthroscopies evaluated by Hjelle *et al*., chondral and osteochondral lesions were identified in 61% patients, 55% grade III and 5% grade IV [40]. The occurrence of cartilage lesions was most frequently observed in the 40s while young adults developed full thickness defects in their 30s [41]. Interestingly, weight bearing medial femoral condyle is the most commonly affected zone accounting for 58% of all cartilage lesions in the knee.

The International Cartilage Repair Society has developed a method for classification of chondral injuries: Grade 0-Normal cartilage, Grade I: Superficial fissures, soft cartilage, Grade II: minor tears, lesions extending less than half of the cartilage depth, Grade III: Deep lesions, lesions extending more than half but not extending into subchondral bone, Grade IV: severe erosion of

cartilage surface penetrating into subchondral bone and exposure of subchondral bone. Cartilage defects can be classified into chondral and osteochondral defects depending upon the depth. Chondral defects can be either partial thickness or full thickness [42]. Full thickness defects which penetrate the subchondral bone are considered osteochondral defects. While repair of chondral defects is limited or non-existent, osteochondral defects have some repair potential attributed to the mesenchymal progenitors recruited from the subchondral bone. In any case, the repair is generally fibrocartilagenous with inferior structural and functional properties with extremely compromised functionality (Fig. 2.1) [42-44]. The treatment modalities are dependent on defect size, depth and location, chronicity, age of patient, previous treatment among others [38].

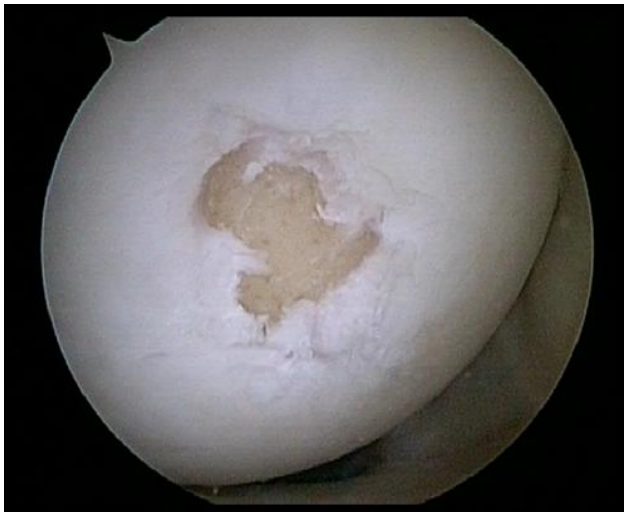

Figure 2.1: An arthroscopic view of the knee joint revealing a full thickness cartilage lesion on medial femoral condyle leading to exposure of subchondral bone. (http://stemcelltreatmentclinic.com/indications/cartilage-defects/)

2.2 Osteoarthritis

Osteoarthritis (OA) is the most prevalent form of arthritis affecting up to 15% of population [45]. It is characterized by progressive loss of articular cartilage and formation of osteophytes which cause chronic pain reducing the quality of life of patients and loss of functional quality of the joints. OA is primarily caused by traumatic events, commonly referred to as post traumatic osteoarthritis (PTOA). Other causes include flawed position of joints, ageing and genetic predisposition [46]. The molecules of interest with respect to OA include agents with a direct

bearing on the matrix turnover such as matrix metalloproteinases (MMPs), aggrecanases and tissue inhibitors of proteinases (TIMPs) in addition to matrix components collagen and GAGs.

Martinek *et al* classified the pathophysiology of OA into three stages [47]. The first stage is characterized by the degradation of matrix network on a molecular level. An increase in the water content is accompanied by loss in aggrecan. This leads to destruction of collagen network and cartilage starts to lose its stiffness. In the next stage, chondrocyte clusters are generated as a result of cloning of cells which get surrounded by newly synthesized matrix. This is a result of an increase in the proliferation and metabolic activity of chondrocytes in order to compensate for the ongoing damage to the ECM. Finally, the cartilage tissue is completely degenerated since the anabolic activities of chondrocytes are unable to match the degradation processes. This stage is further characterized by subchondral bone cysts, formation of osteophytes and osteochondral nodules.

As mentioned before, an imbalance in the anabolic and catabolic processes in the articular cartilage leads to an increase in degradation as well as synthesis in the cartilage matrix (Fig. 2.2). The overexpression of matrix degrading enzymes results in steady loss of collagen and proteoglycans which causes the chondrocytes to proliferate and increase synthesis of matrix components. The disease progresses because these repair attempts are unable to balance ongoing cartilage degeneration [48]. The inflammatory cytokines-such as IL-1, IL-17 and TNF-α- lead to an increase in the expression of MMPs accompanied by a decrease in the expression of their inhibitors [49]. The damaging cytokines are released by synovial cells as well chondrocytes themselves [50]. Simultaneously, anabolic factors such as IGF-1, TGF, FGF and BMPs attempt to rescue matrix degradation by inciting ECM synthesis [48]. The degradation is further aggravated by the action of degradation fragments of fibronectin and collagen type II [51].

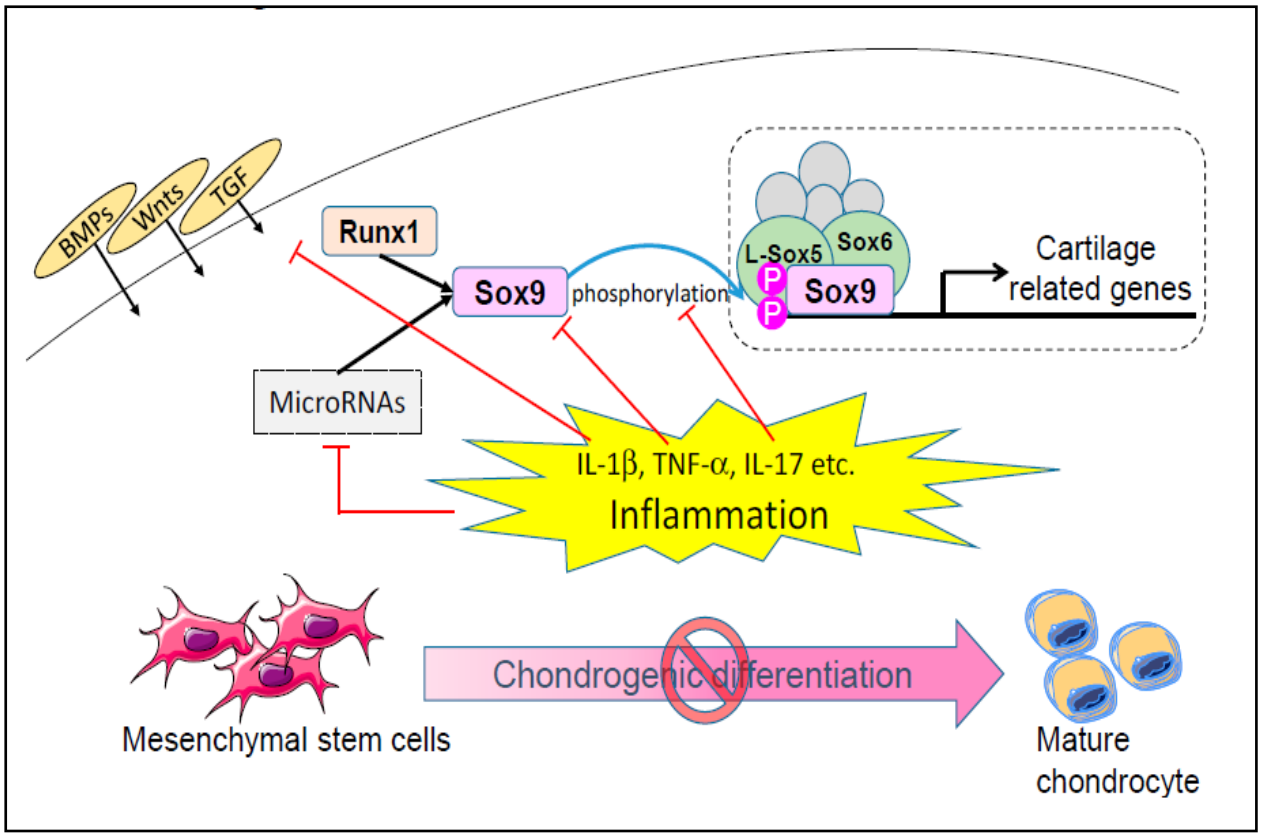

Figure 2.2: Schematic diagram depicting the inhibitory role of inflammatory mediators IL-1β, TNF-α and IL-17 in chondrogenic differentiation of BMSCs. BMP-Bone morphogenetic protein, TGF-Transforming growth factor, Runx-Runt related gene, Sox-SRY box. [*Image copied from Kondo et al*- 52]

OA also has a bearing on the BMSCs present in bone marrow. Reduction in the proliferation rate of BMSCs was reported by Murphy *et al* [53]. However, other studies suggest that the inhibitory effect of OA on BMSC proliferation is not absolute [54]. The effect of OA on the chondrogenic potential of BMSCs is equally controversial. CD105+/CD166+ population of BMSCs isolated from OA patients were shown to have similar chondrogenic potential as healthy BMSCs [55] [56]. In another study, BMSCs from OA patients had reduced chondrogenic potential and poor GAG deposition [53]. Kruger *et al* showed that synovial fluid from patients with OA delayed but did not completely inhibit the chondrogenic of BMSCs [57]. However, the limited literature on the effect of OA on functional behaviour of BMSCs suggests that OA BMSCs do not lose the

chondrogenic potential completely [58]. Progenitor cells also tend to migrate into the defect through the cracks in the subchondral bone and carry out a limited repair [59]. The inflammatory mediators released during OA are also detrimental for BMSC behaviour. The prominent mediators include IL-1α, IL-1β and TNF-α [60, 61]. The inhibitory action of IL-1β and TNF-α in inhibiting chondrogenic differentiation of BMSCs was shown to be proceeding through NF-κB mediated pathway leading to suppression of Sox-9 through TGF-β II receptor suppression accompanied by increase in Smad-7 levels and inhibition of Smad 2/3 [62, 63]. BMSC differentiation is also suppressed by another major inflammatory cytokine IL-17 mediated by inhibition of Sox-9 phosphorylation [64]. Il-1β also alters the expression of miRNAs-such as miR-140, miR-145, miR-199a- involved in chondrogenic differentiation of BMSCs leading to suppression of chondrogenic differentiation of BMSCs [62, 64]. Presence of inflammatory mediators also enhances calcification due to preferred osteogenic differentiation of BMSCs leading to increase occurrence of osteophytes and ectopic calcifications [61, 62]. Application of anti-inflammatory agents in such cases is expected to maintain functional and cellular behaviour of BMSCs leading to better cartilage repair outcome.

2.3 Bone marrow stem cells (BMSCs)/Mesenchymal stem cells (MSCs)

2.3.1 Introduction and history

Bone marrow (BM) is a complex interacting system mainly comprised of hematopoietic, endothelial and stromal components [65]. The hematopoetic compartment of BM is made up of multipotent stem cells, committed progenitor cells of myeloid and lymphoid lineages and maturing cells [66]. A wide array of cells including macrophages, adipocytes, osteogenic cells and reticular cells make up the marrow stroma [67]. *In vitro* culture of marrow stroma is comprised of a mixture of fibroblastic cells, macrophages, adipocytes, endothelial cells, and smooth muscle cells [68]. In addition, two distinct populations of stem cells co-exist in marrow, the hematopoetic stem cells (HSCs) and BMSCs [69]. The matrix is primarily composed of fibronectin, collagen, vitronectin and tenascin [70]. In addition to soluble factors such as stem cell factor, granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor, stroma is also characterized by presence of adhesion molecules such as VLA-1-6 [71]. BMSCs secrete several trophic factors with paracrine effects. In addition to

differentiation, the trophic factors, including growth factors, adhesion molecules and cytokines have been shown to alter tissue microenvironment leading to tissue repair and regeneration [72].

Stem cells can be defined as cells with ability to self renew and proliferate indefinitely without differentiating (Fig. 2.3) [70]. Friedenstein *et al* were the first to identify stem cells (progenitor cells) in hematopoetic microenvironment of bone marrow in 1970s [73] although Julius Cohnheim in 1867 had proposed the existence of non-hematopoetic stem cells when he observed the presence of fibroblast like cells in repairing wound which were derived from circulation [74]. The cells were recognized as "mesenchymal stem cells" by Caplan in 1991 [75]. The bone marrow niche for BMSCs was proposed by Owen in 1985 who suggested that stromal cells reside in a niche of their own with self renewal property and capability to generate mature stromal cells [75]. These cells originate from the mesenchyme, which can be defined as loose connective tissue, mostly derived from mesoderm of developing embryo [76]. The trilineage potential of BMSCs was demonstrated by Pittenger *et al* in 1999 [77]. BMSCs are characterized by their intrinsic self-renewal capacity i.e. the ability to remain in an undifferentiated and unspecialized state after each division. *In vitro* cultures of BMSCs can proliferate and maintain differentiation potential until 40 population doublings [78, 79]. In adult tissues, MSCs carry out the function of replacing and regenerating local cells that are lost to normal tissue turnover, injury or ageing. In addition, they maintain hematopoiesis by providing growth factors and promoting cell-cell and cell-matrix interactions owing to several cytokines released by them [69]. As shown by Pittenger *et al*, these cells are extremely rare and form a mere 0.01 to 0.001% of the mononuclear fraction isolated from bone marrow [80] which is primarily made up of macrophages, adipocytes, osteogenic cells and reticular cells [68]. Several studies carried out in the recent past have indicated a perivascular niche for MSCs and that they exist as pericytes at several locations throughout body [81, 82]. Chagastelles *et al* showed close proximity between pericytes and MSCs which had similar expression of Stro-1, α-smooth muscle actin, CD44, CD90 and CD105 [83]. In addition to serving as a pool of cells for regeneration and repair of tissues during an organism's lifetime, MSCs also play several important roles owing to their paracrine effects resulting from secretion of cytokines, growth factors, chemokines, angiogenic and immunosuppressive factors [84].

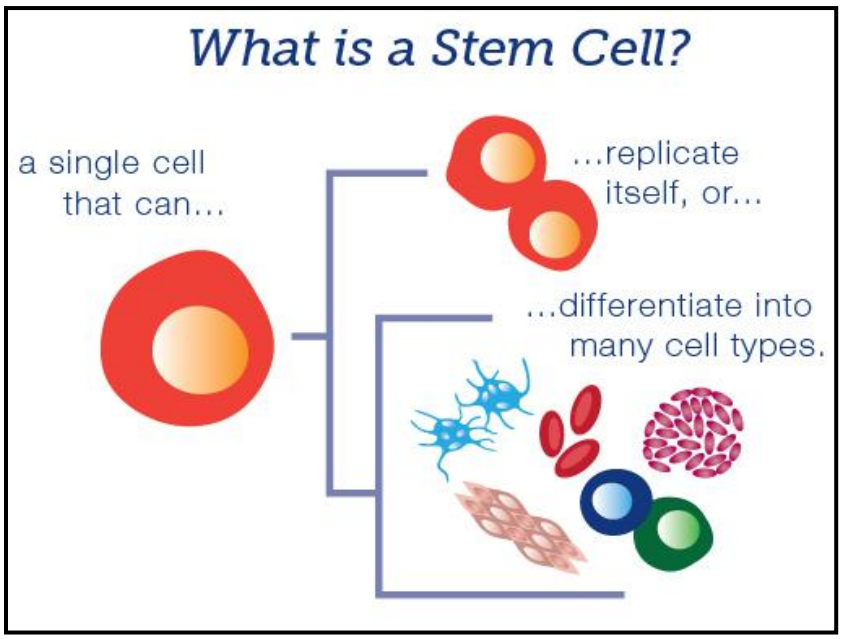

Figure 2.3: Schematic representing the properties and role of stem cells. [*Copied image* -67]

2.3.2 Isolation of BMSCs

Initially contaminated with cells from hematopoetic origin, the cultures are almost 100% pure by second passage [85]. Upon isolation, cells form foci of 2-4 cells and grow as colonies in P0. The cells may take variable amount of time for adherence depending upon method of isolation, age of the donor and other factors like source of cells (fast for suspended cells and slower for cells derived from explants). The proliferation begins only after a dormant phase of 2-4 days after adherence [86]. Despite being “stem cells”, MSCs possess limited proliferative potential and can be passaged for 25-40 population doublings over 8-15 passages. The signs of ageing include reduced proliferation potential and change of morphology to large, loosely packed cells [87-89]. Tuan *et al* showed that cells in trabecular bone chips (explants) reacted positively with CD73, Stro-1 and CD105 antibodies while were negative for CD34 and CD45 suggesting presence of multipotential cells in bone [90]. The population isolated from explants is mostly homogenous and 100% positive for CD73, CD105 and Stro-1 unlike the population isolated from bone marrow which is more heterogenous. It will be interesting to explore if this difference has an implication on the differentiation potential of these cells as well.

BMSCs can be isolated from bone marrow (BM) using several methods including density gradient centrifugation, direct plating and explant culture of bone chips. Since stromal matrix is primarily composed of collagen type I (Col-I) and IV (Col-IV), collagenase enzyme is used for isolation of stromal BMSCs [91, 92]. Collagenase enzyme has been typically used to remove soft connective tissue from the trabecular bone for the isolation of progenitor cells from subchondral regions of bone which include the marrow stroma and bone lining cells [93]. The efficiency of digestion process depends upon the conditions used during collagenase treatment. Collagenases used in R&D are crude mixtures of interstitial (E.C.3.4.24.7) and neutrophil (3.4.24.34) collagenases. The former is a type MMP-1 while the latter is a type MMP-8 enzyme. The enzyme isolated from *C.histolyticum* typically contains a mixture of collagenase, clostridiopeptidase and non-specific protease (IUBMB- www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/24/3.htm). Although, several protocols are used for isolation of BMSCs using collagenase digestion, the yield is generally higher when enzymatic digestion is used as a method of isolation as opposed to BM aspiration [94]. While some believe that enzymatic isolation alters the characteristics of BMSCs [95], others claim that these cells are phenotypically similar to cells obtained from BM aspirate [94, 96]. Expansion of BMSCs has been shown to have a negative impact on differentiation potential with increasing passages [97]. The media used for *in vitro* expansion of BMSCs is typically comprised of a basal media containing glucose, amino acids, ions, and fetal animal sera [98]. An ideal culture of BMSCs would be characterized by a). Maintenance of original phenotypic and functional characteristics, b). Indefinite proliferation, c). Multilineage differentiation potential [70].

In vitro characterization of BMSCs is important in order to understand the cellular and functional behaviour of BMSCs (Fig. 2.4). *In vivo* analysis is difficult owing to low frequency and lack of markers for their *in vivo* localization [70]. The culture systems primarily aimed at identification of BMSCs may be classified into CFU-f assay, analysis of BM stroma, and culture of BMSCs [70]. Clonal analysis has shown that BMSC populations are a heterogenous mixture of tri-, bi- and unipotent subpopulations with varying levels of lineage commitment [87, 99, 100]. BMSCs also possess limited transdifferentiation capacity dependent on culture conditions [101].

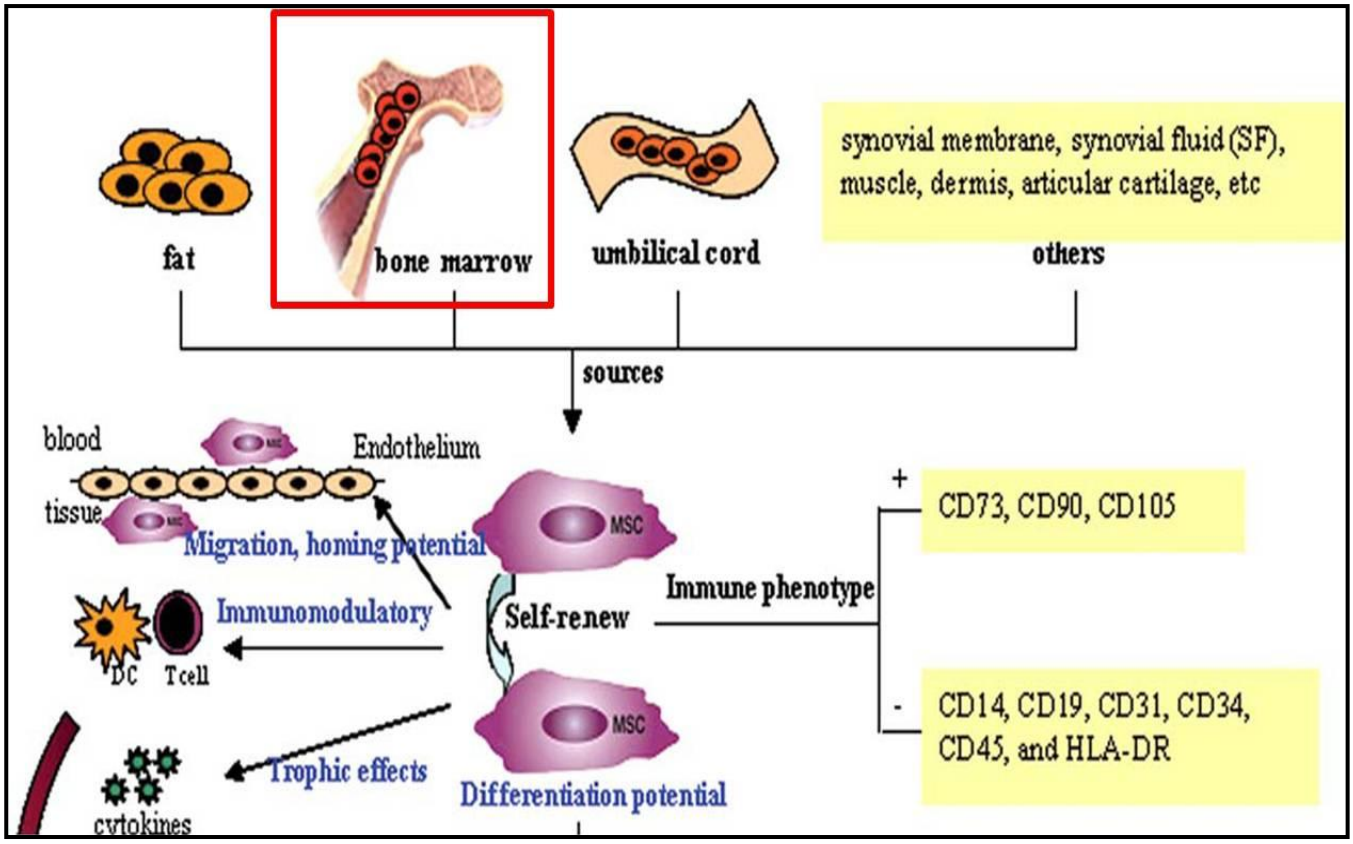

Figure 2.4.: *In vitro* characterization of BMSCs depicting their surface marker profile and differentiation potential. [*Image copied from Si et al* - 102]

Since the isolated BMSC population is highly heterogenous with regards to morphology, physiology and expression of markers, International Society of Cell Therapy (ISCT) in 2006 proposed a standard set of criteria to identify and characterize these cells *in vitro* [15]. First, BMSCs must adhere to tissue culture plastic, a property which is commonly applied for their isolation. Second, more than 95% BMSC population must express a specific set of membrane molecules such as CD105, CD73 and CD90 in addition to lacking the expression of hematopoetic markers like CD34, CD45, CD14, CD11 and HLA-DR as determined by flow cytometry. Interestingly, CD105 was originally described as SH2 antibody raised against human BMSCs which reacted with epitope present on TGF-β receptor endoglin CD105 [103]. Similarly, SH3 and SH4 antibodies recognize membrane bound ecto-5'-nucleotidase CD73 [104]. It is important to note here that due to lack of an exclusive marker of BMSCs, their in-situ localization still eludes us [105]. Trilineage differentiation potential is the final ISCT criterion according to which BMSCs must differentiate into cells of osteogenic, chondrogenic and adipogenic phenotype upon

suitable stimulus. In order to differentiate BMSCs, a whole range of growth factors such as FGF, TGF and BMP, hormones such as dexamethasone, vitamins such as ascorbic acid and chemicals like β-glycerophosphate play an important role. Apart from the growth factors and cytokines, signaling molecules like Wnt, Notch, Runx2, Ihh also contribute in determining the fate of BMSC differentiation [106]. The multilineage differentiation potential is evidenced by the role these cells play in remodeling of bone under normal physiological conditions and fracture repair. The long bones in the body grow by means of a cartilaginous intermediate. The adipogenic potential of BMSCs is demonstrated when they increasingly lose bone forming potential with advancing age and more and more tissue in bone is replaced by adipocytes [107].

2.3.3 Colony forming unit assay (CFU-f)

In addition to above properties, clonogenic capability is another property used to characterize BMSCs [108, 109]. CFU-fs have been described as rapidly adherent, non-phagocytic clonogenic cells with ability of extended proliferation *in vitro* [110]. Cells from BM are seeded at low density and individual colonies are scored as an indicator of clonogenic potential or stemness of a culture since each colony is presumed to have been cloned from a single precursor cell [65]. Several groups have used the assay to suggest that CFU-fs occur at a frequency of one in 10^4 to 10^5 of marrow mononuclear cells [65]. Upon isolation from bone marrow, the adherent cells have a heterogenous morphology due to which it is difficult to clone them [87]. It is therefore ideal to carry out the CFU-f assay at passage 1. It is essential to carry out the CFU-f assay at low density since the colonies grow in isolation at low density and represent clonal expansion of a single CFU-f or mesenchymal progenitor cell thus being a measure of the number of BMSC population [111]. Friedenstein was the first to propose the concept of colony forming units and demonstrated that each colony in BMSC culture was derived from a single fibroblastic colony forming cell. These CFU-fs are heterogeneous in morphology [87] as well as differentiation potential as confirmed by several other authors [112]. Cells in a colony are not necessarily genetically similar and have been shown to be heterogeneous in their lineage commitment and multilineage differentiation potential *in vitro* as well as *in vivo* [113, 114]. The CFU-f forming capability of an BMSC culture has been shown to be influenced by several factors including media composition but is also an indicator of bonafide differences existing *in vivo* [69].

2.3.4 BMSC surface marker expression

Stro-1 is an important marker which has found application in enriching the mesenchymal precursors [115]. Expression of CD34 is lacking in culture expanded BMSCs and cells isolated from CFU-fs [80]. However, a low level of CD34 has been detected in BMSCs isolated from some species, making it a controversial negative marker [116]. It is likely that CD34 expression is lost upon *in vitro* expansion. The CFU-f precursor has been shown to be positive for expression of Thy-1 (CD90), integrin family (CD29, CD49) and VCAM-1 in addition to receptors for PDGF, IGF and EGF [117].

It is critical to carry out the characterization assays in early passages as long term culture of BMSCs have been shown to have detrimental effect on their fundamental phenotype. This becomes an important consideration in a scenario where *in vitro* characterization of BMSCs is supposed to be reflective of their *in vivo* phenotype. *In vitro* culture and expansion has been shown to affect the stemness, senescence and genetic stability [118] as well as expression of cell surface markers and differentiation potential. It is also important to consider that application of anti-human antibodies for characterization might lead to variation in expression of these markers [119].

2.3.5 Heterogeneity of BMSCs

As mentioned before, BMSC cultures are highly heterogeneous with regards to morphology and function. The *in vitro* cultures are generally composed of a population containing both single stem-cell like cells and progenitor cells with different lineage commitment [120]. In this heterogeneous population, only a fraction of cells are truly multipotent and possess the capability of self renewal [121]. The problem is further compounded due to presence of donor-to-donor and intra-population heterogeneity [77]. Phinney *et al* observed striking heterogeneity in growth rate, alkaline phosphatase levels and osteogenic potential [77]. Donor dependent heterogeneity is attributed to several factors including sampling bias, age of donor and method of culture [99, 122-124].

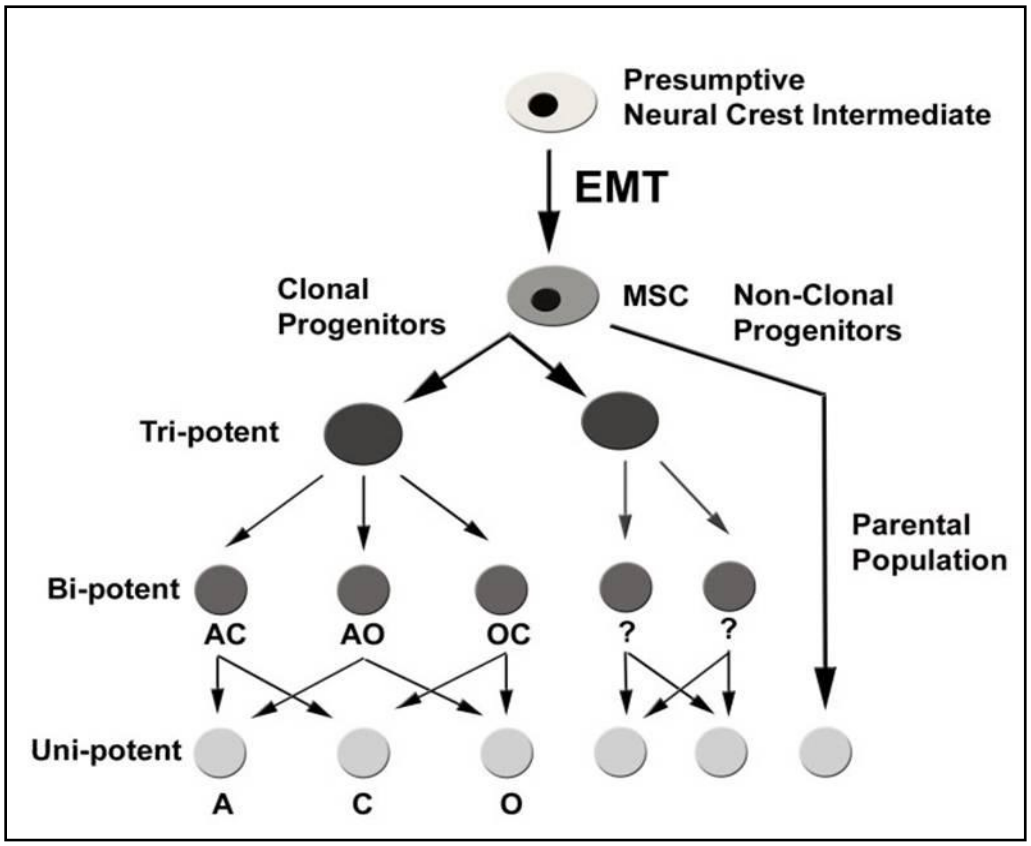

Figure 2.5: Schematic illustrating the hierarchical program of lineage specification and evolution of tri-, bi- and unipotent progenitors [*Image copied from Phinney at al*-77].

BMSC phenotype was identified to be made up of 113 transcripts and 17 proteins [125]. Since BMSCs have been shown to lose multipotential differentiation capacity without a corresponding loss in surface phenotype, a combination of assays are needed to evaluate growth, proliferation and functional behaviour including differentiation potency [87, 97, 126]. In their pioneer work, Muraglia *et al* [100] analysed 185 huMSC clones to demonstrate a dominance of tri-potent, osteo-chondrogenic and osteogenic progenitor. This indicated the maximum propensity towards osteogenic lineage while the BMSC clones were more likely to lose adipogenic commitment. This phenomenon is recognized as linear progression which follows a hierarchical program of lineage specification (Fig.2.5). In a later study, approx 50% of population was found to be truly tripotential cells while 5% were lacking differentiation potential. Tripotent cells were also characterized by high proliferation potential and low apoptosis [127].

2.3.6 Differentiation of BMSCs

2.3.6.1 Chondrogenic differentiation

While osteogenic potential of progenitor cells was demonstrated for the first time by Friedenstein in his pioneer work, chondrogenic potential was revealed much later in 1998 in critical works of Johnstone and Mackay [128, 129]. Johnstone *et al* illustrated that chondrogenesis can be induced in BMSCs in aggregate cultures in the form of pellet which has since become the gold standard to differentiate BMSCs into cartilaginous phenotype [128]. It is now known that pellet cultures provide the critical cell-cell and cell-matrix interactions which resemble microenvironment in precartilage condensation bud and promote chondrogenesis while maintaining chondrogenic phenotype. Mackay *et al* provided evidence that upon stimulation by TGF-βIII and dexamethasone, the stem cells differentiated and acquired chondrogenic phenotype. In this important study, Mackay *et al* found that cells failed to differentiate into chondrocytes when either TGF or dexamethasone was omitted from the media emphasizing the roles of these factors. In fact, one of the earliest activities of TGF-βIII was found to be its capability of inducing chondrogenesis in rats [128, 130, 131]. In addition, chondrogenesis was found to be superior when TGF-βIII was used instead of TGF-βI [132]. Dexamethasone, a synthetic glucocorticoid supports viability, inhibits hypertrophy and increases the expression of Col-II, aggrecan and COMP [128, 129, 133]. Ascorbate is responsible for hydroxylation of proline and lysine residues required for collagen synthesis [134]. On comparison of DMEM containing low v/s high concentration of glucose (1g/l v/s 4.5 g/l), increase in pellet size was higher in the case of latter. This effect could be due to protective action of higher concentration of glucose against cell death thus increasing viability. Moreover, a hypoxic environment is created due to respiratory inhibition caused by high concentration of glucose which promotes chondrogenic differentiation. High glucose also alters cell metabolism and makes cells less susceptible to apoptosis [129]. Since cartilaginous matrix was not observed in pellets from all donors when cultured for a period less than three weeks, this period has been considered as the most optimum duration for inducing chondrogenic differentiation [129]. The increase in the size of the pellets at the end of this period was found to be due to accumulation of matrix and not increase in cell number since DNA content was found to be fairly constant for the period of differentiation. Although Col-I is not found in normal articular cartilage, a peripheral zone of Col-I is detected in pellets where cells are flattened and resemble perichondrium [129].

In their study on the events of chondrogenic differentiation in pellets, Yoo *et al* found that after an initial reduction in size, matrix synthesis kicks in which leads to a gradual and constant increase in the pellet size thereafter [135]. Sekiya *et al* analyzed 12000 genes during chondrogenesis and found marked changes in the expression of matrix proteins and transcription factors such as Col2a1, Agc1, COMP, Col-IX, Col-X, Sox-9, Sox-5, Sox-6, BMPs, PTH, PTHrP etc indicating their importance in chondrogenic differentiation of BMSCs [136].

Pellets are highly heterogeneous tissues possibly due to the radial expansion of the cells which exert biomechanical forces on the tissue. Chondrogenic differentiation requires presence of interaction between cells and absence of adhesion to substrate [137]. Differentiation starts simultaneously at several sites and spreads gradually throughout aggregate [138]. The size of aggregates starts to increase 203 times after first week. A 2-3 cell layer deep outer layer resembling perichondrium is made up of flattened, undifferentiated BMSCs. The matrix in this zone is rich in Col-I and poor in Col-II and proteoglycans. Below this, the chondrocytes are rounded and resemble the native cartilage. They secrete a matrix that is rich in Col-II and aggrecan. The innermost core is last to differentiate and is normally comprised of poorly differentiated or necrotic zone [128, 129, 135]. Since PCR can detect mRNA long before the expression of protein, a histopathological analysis of pellets is carried out to determine the phenotype. Safranin-O staining remains to be the simplest and most efficient method to detect the presence of glycosaminoglycans (GAGs) in matrix. A more extensive immunochemical study to detect the presence of Col-I and Col-II is carried out to generate a complete profile of chondrogenic differentiation [139, 140]. TGF-β superfamily plays an important role in regulation of chondrogenesis and TGF-β, BMP-2 and GDF-5 have been implicated in chondrogenic regulation [141-143].

TGF-β has been reported to be the most essential factor required for chondrogenic differentiation. TGF-βIII induced ERK1/2 pathway was activated for 1-14 days during chondrogenesis suggesting its importance in chondrogenic differentiation of BMSCs [144]. This pathway has a significant bearing on the expression of collagen type II in pellet cultures of BMSCs [144]. It is also believed to inhibit hypertrophy, an undesirable phenomenon during chondrogenesis [129]. In

a study carried out using chick wing bud mesenchymal stem cells, Jin *et al* showed that expression of Wnt 5α was upregulated in the presence of TGF-βIII by modulating PKC-α and p38 mitogen activated protein kinase (MAPK) activity. Activation of Wnt 5α led to the upregulation of synthesis of adhesion molecules like fibronectin and integrin α5 [145]. A crosstalk between Wnt, SMAD, MAPK and pathways induced by PTHrP and Ihh regulate the genes required for chondrogenesis including Sox9 which is master regulator of chondrogenic determination of BMSCs. Aggrecan gene expression on the other hand was shown to be dependent on a cross talk between Smad, ERK1/2 and MAPK pathways (Fig.2.6) [146]. The potential of BMSCs for cartilage repair was reported for the first time by Wakitani *et al* in 1994. BMSCs dispersed in collagenous matrix when implanted in full thickness defects in rabbits formed cartilage by two weeks [147].

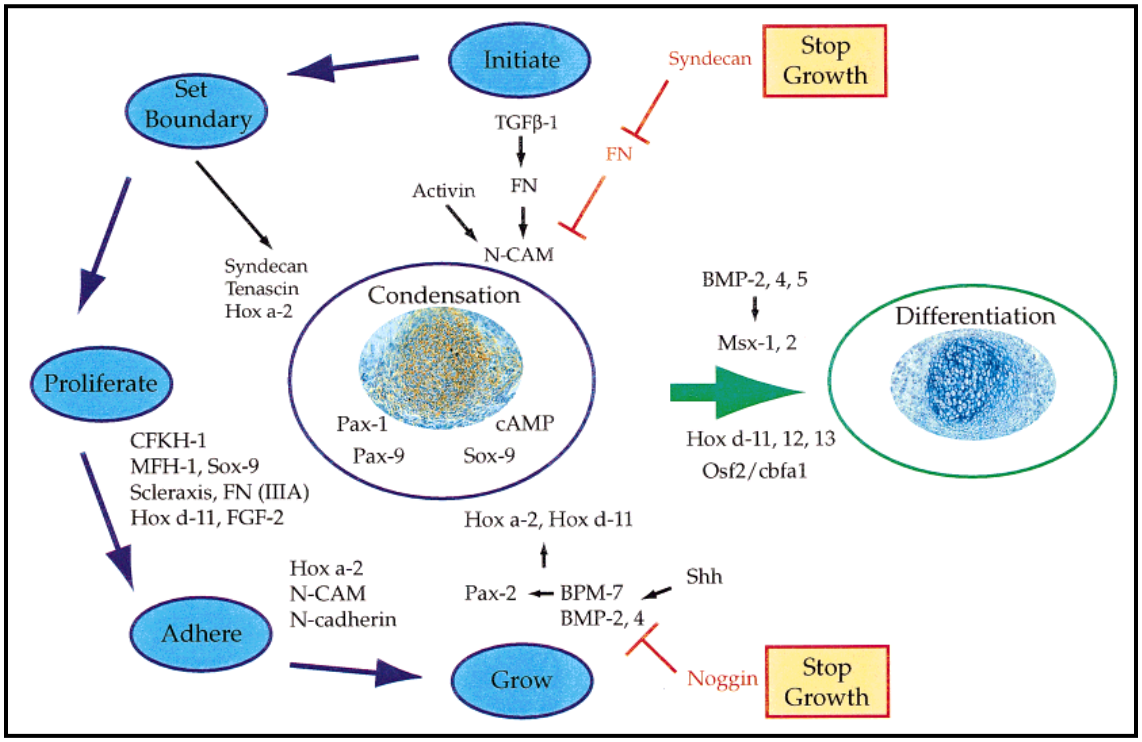

Figure 2.6: Schematic of stages in condensation phase of embryonic limb formation. Adapted from Maeda *et al* [67].

Differentiation of BMSCs into cells of cartilaginous phenotype in pellets under *in vitro* conditions and during formation of repair tissue *in vivo* closely resembles the events of formation of limbs during embryonic development [148]. The cells from lateral plate mesoderm migrate to the site of skeletogenesis, characterized by expression of hyaluronan and upregulation of cAMP.

In the next stage, cell-cell interactions are encouraged due to N-cadherin and N-CAM under the influence of TGF-βIII. Syndecan and Tenascin set limit to this condensation where cells proliferate further under the influence of cfkh-1 and mfh-1. Sox-9 induces the expression of col2a1 gene at this stage. In order to induce differentiation, expression of proliferation and adherence related genes are downregulated under influence of BMPs, noggin and Syndecan. In a series of events influenced by multiple effector molecules such as Ihh, PTH/PTHrP, Sox-5/6/9, BMPs, Hox, and pathways including ERK1/2, Smad and MAPK, mature chondrocytes are formed which lay down a matrix rich in Col-II and aggrecan (Fig. 2.7) [146]. According to some reports, extracellular matrix components modulate differentiation and regulate BMSC adhesion. Matrix metalloproteases and tissue specific inhibitors of metalloproteases have been implicated in regulation of commitment by BMSCs [149].

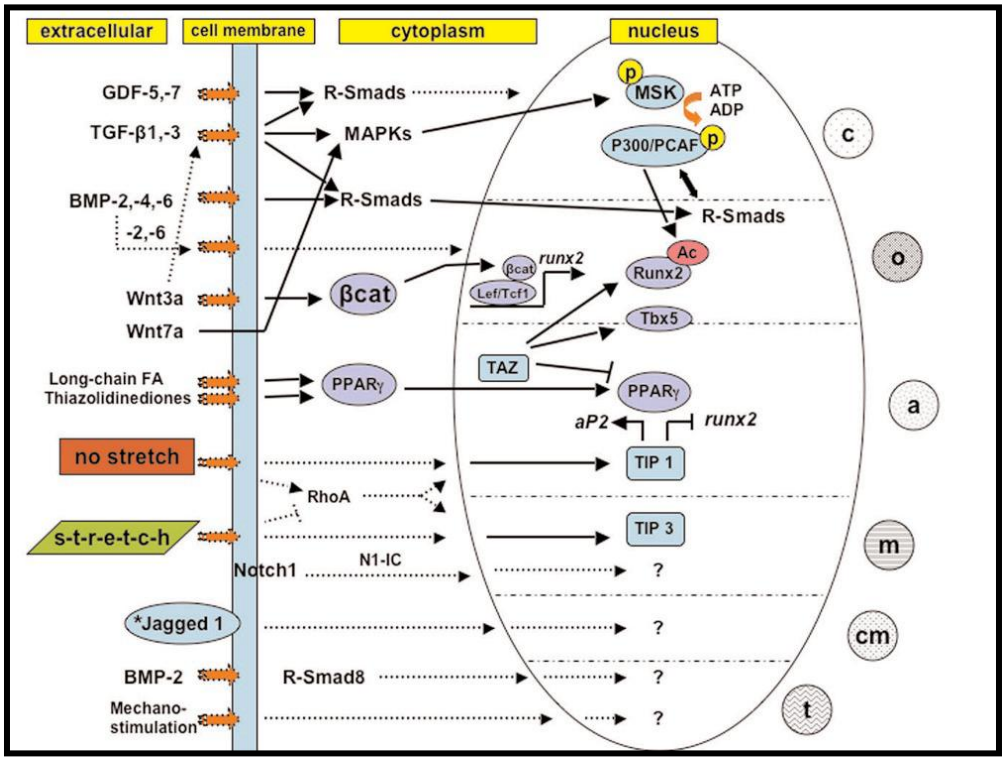

Figure 2.7: Molecular regulation of mesenchymal stem cell differentiation programs. Differentiation is transduced as a result of interaction between extracellular molecular signaling and mechanical inducers and putative receptors, channels, and/or other cell-surface-associated mechanisms. The downstream lineages arise from crosstalk of signaling pathways, including that between distinct mitogen-activated protein kinases (MAPKs) and R-Smads. GDF- growth and

differentiation factor; TGF- transforming growth factor; BMP, bone morphogenetic protein; FA- fatty acid; βcat- β-catenin; PPAR- peroxisome proliferator-activated receptor; MSK- mitogen- and stress-activated protein kinase; PCAF- p300/CBP-associated factor; Ac- acetyl; c- chondroblast; o- osteoblast; a- adipoblast; m- myoblast; cm- cardiomyoblast; t- tenoblast. [*Image copied from Kolf et al*-150].

2.3.6.2 Osteogenic differentiation

Friedenstein had first demonstrated the osteogenic potential of CFU-f isolated from bone marrow. Bone tissue formation was observed in diffusion chambers filled with CFU-fs and implanted in donor animals [138]. *In vivo*, osteoblasts and bone lining cells are believed to originate from multiple sources including Nestin(+) endosteal BMSCs [151], periosteal stem cells, pericytes and other cells from vascular walls, and common endothelial/osteoblast progenitors that may be in the circulation [152-154]. *In vitro* osteogenic assays can be derived from fetal calvarial cells, BMSCs and cells originating from bone explants [155]. Osteogenic differentiation of BMSCs is typically carried out in a media comprised of α-MEM containing dexamethasone, ascorbate and β–glycerophosphate (GP). BMP-2 and 7, FGF-2 and PGE2 are known stimulators of osteogenesis [156, 157] while LIF, oncostatin, EGF and calcitrol are some of the inhibitors [67, 158, 159]. It is characterized by alteration in morphology to polygonal, active basophilia in the cytoplasm, increase in alkaline phosphatase (APase) activity and deposition of calcium (Ca) rich matrix which is illustrated by Alizarin Red S and vonKossa staining [160]. The matrix deposition could be nodular as in rats or uniform as in humans [78]. In a study carried out by Cheng *et al*, physiological concentration of dexamethasone was found to be an absolute requirement for osteogenic differentiation [161]. Dexamethasone enhances self-renewal and survival and induces the expression of APase (initially, then decreases it) and Ca mineralization mediated by induction of Runx 2 and osterix [155] [162]. β-glycerophosphate has no effect on proliferation and increases matrix mineralization by donating inorganic phosphate [163]. Ascorbate promotes collagen synthesis [162]. During embryonic development, formation of long bones takes place by endochondral ossification. Post condensation and overt differentiation into chondrocytes, Ihh induces further differentiation characterized by expression of Col-X. BMPs upregulate expression of osteogenic inducers cbfa-1 and Runx-2. This is accompanied by activation of MMPs which degrade the cartilage matrix. Col-I, osteopontin and

osteocalcin genes are upregulated leading to synthesis of bone matrix. In the last stages, VEGF mediated angiogenesis leads to vascularization. MMP-9, 13 and 14 bring about further degradation of non-ossified matrix which is then replaced by calcified bone matrix. Under *in vitro* conditions, osteogenic differentiation is comprised of an initiation phase which is characterized by increase in proliferation followed by enhanced expression of ATPase and Col-I. In the maturation phase, matrix mineralizes primarily with hydroxyapatite aligned with collagen fibers. Calcium deposition is an important event in osteogenesis and indicates terminal differentiation. Smad pathway mediated by Runx2 and BMPs is the most important pathway underlying osteogenic differentiation [36, 164]. Several pathways have been implicated in osteogenic differentiation of BMSCs. The ERK pathway is activated in early stages by binding of laminin 5 to cell which leads to phosphorylation of osteogenic transcription factor cbfa-1 [165]. Activation of JNK pathway occurs later once the ECM is in mineralization phase [166]. Other factors which promote osteogenesis include Wnt3A and Cadherin 11 which increase the expression of tissue growth factor and alkaline phosphatase respectively. Sortilin and FGF-2 are needed for mineralization of matrix [167, 168].

The osteogenic differentiation can be characterized by evaluating alkaline phosphatase and mineral deposition as end points (Fig. 2.8). The primary alterations during osteogenesis are change in morphology of cells from spindle to oblong and expression of non-mineralized matrix rich in collagen [155]. ALP activity increases by 2-6 fold when cultured in presence of dexamethasone, BMP-2 or vitamin D3 [169, 170]. Dexamethasone is the most potent osteogenic stimulator which is an agonist of glucorticoid receptors found on BMSCs [171]. Exposure to dexamethasone stimulates cuboidal morphology, ALP mRNA and suppresses bone sialoprotein [172, 173]. A concentration of 10 nM of dexamethasone is more conducive for stimulation of osteogenic phenotype [174, 175] since concentration of 100 nM was found to inhibit expression of osteopontin [175, 176]. In the first phase of development spanning 1-2 week, slow proliferation of cells is accompanied by expression of ALP activity, and assembly of collagen matrix along with expression of bone specific marker genes. The second phase occurs in week 2 or 3, calcium phosphate is expressed causing matrix mineralization [177-179]. Presence of Pi is necessary during this phase and inorganic phosphate is usually provided by addition of 3mM Pi or 5-10mM disodium beta glycerophosphate [180]. The functional endpoint of advanced cell

differentiation is the mineralization. The calcium component of the matrix can be analysed by alizarin red staining while Von Kossa staining can be used to detect the presence of phosphate component. The mineral formed during osteogenesis may be substituted hydroxyapatite or non-hydroxyapatite. The former exists with collagen fibrils and appropriate ratio of calcium and phosphate. The latter is characterized as cell-associated precipitate with higher calcium/phosphate ration than collagen [163, 177, 181, 182].

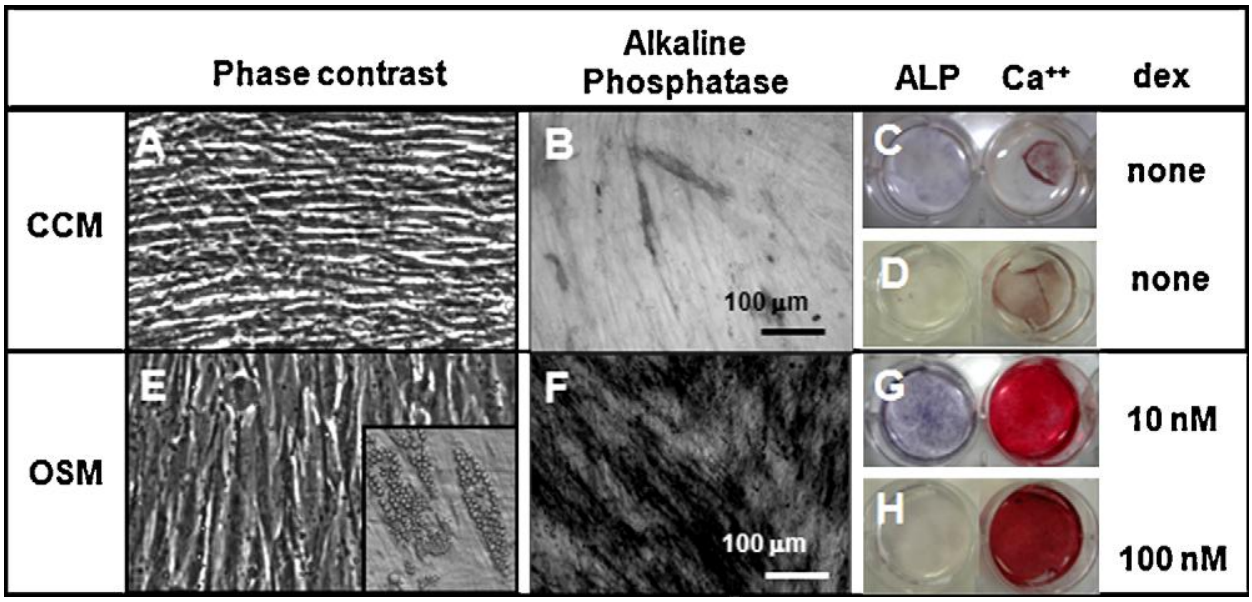

Figure 2.8: Representative osteogenesis cultures cultured in control media (CCM) and osteogenic media (OSM) containing dexamethasone. The osteogenic phenotype was represented by cuboidal cells and detected by Alizarin Red S and alkaline phosphatase staining. [*Image copied from Hoemann et al*- 155].

The mechanisms for *in vitro* differentiation of BMSCs have been explored in various species and similar protocols can be applied for osteogenic differentiation of BMSCs in presence of dexamethasone, vitamin D3 and BMP-2. Osteoblastic differentiation was accompanied by upregulation of 83 proteins and downregulation of 21 proteins [183]. Overexpression of cbfa-1/Runx2, ostrix and lipoprotein related factor 5 induce osteogenic differentiation [184-186]. Kassem *et al* compared the EGF and PDGF mediated osteogenic pathways and found that EGF and PDGF utilized more than 90% signaling proteins while PI3K was only activated by PDGF alone [187].

2.4 Bone marrow stimulation for cartilage repair

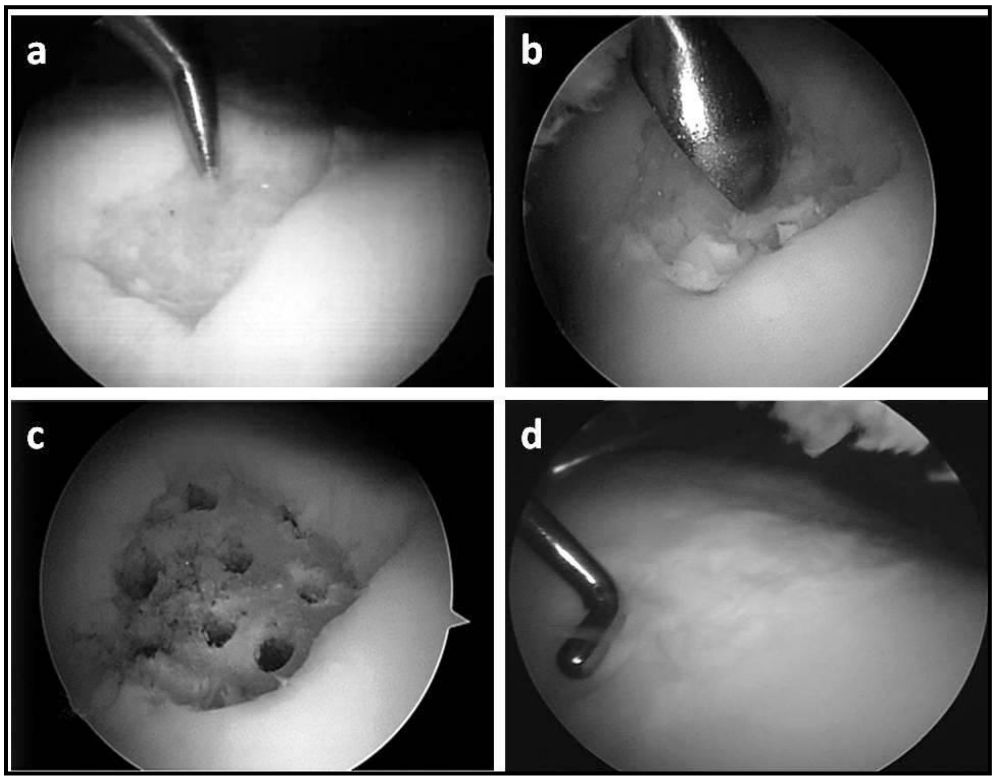

Figure 2.9: Stages in BMS procedure. A. Arthroscopic images of a trochlear defect. B. Debridement of the calcified layer in the defect using a curette. C. BMS procedure using an awl to create multiple perforations in the subchondral bone. D. Second look at 1.5 years after BMS showing asymptomatic lesion in the same patient. [*Image copied from Gill et al*-188].

Amongst several techniques existing for cartilage repair which include autologous chondrocyte implantation, mosaicplasty and osteochondral autograft/allograft transplantation (OATS), bone marrow stimulation (Fig. 2.9) by means of drilling, abrasion arthroplasty or microfracture remains most popular, especially for young patients with smaller defects. While drilling was introduced by Pridie in 1959 [189] and continued to be a widely accepted method for decades, Microfracture (MFX) has become more prevalent ever since being introduced by Steadman in 90s [16, 190]. The spontaneous repair of cartilage can proceed through intrinsic or extrinsic mechanism. The former relies on the limited proliferation capacity of chondrocytes in the region adjacent to defect and is usually insufficient for repair. In the latter, new connective tissue is

formed from subchondral bone progenitor cells which undergo metaplastic changes to form a cartilage repair tissue [191]. Although small foci of hyaline cartilage are formed over the intertrabecular surfaces exposed due to erosion of subchondral bone, it is insufficient to develop a repair tissue [192]. Instead, frank defects created in subchondral bone by means of drilling [189] or MFX [16] lead to generation of a cartilaginous repair tissue. Both events rely on the subchondral bleeding caused by fracture of subchondral bone and recruitment of marrow derived progenitor cells which repair cartilage and bone in a sequential manner [192].

Preparation of cartilage defect during human surgeries as well as creation of an animal model is an important first step to ensure success of this procedure. The cartilage defect must have clean 90 degrees wall of normal cartilage and all the calcified cartilage must be removed taking care not to injure the subchondral bone. MFX is performed with a specialized awl that is used to create 1-1.5mm holes 3-4 mm apart in the subchondral bone. In the case of microdrilling a drill burr with diameter of 1-1.5 mm is used to create drill holes. While the former procedure leads to bone compaction and necrosis adjacent to holes, the latter generates a lot of heat [193]. However, accompanied by cooling irrigation system, microdrilling is a favorable procedure since it removes bone debris and creates clean holes thus improving the repair outcome [11, 12, 16, 189]. On comparison of one day postoperative acute events in a rabbit model, MFX brought about heavy fracturing of bone matrix and dense, compact bone was observed surrounding MFX holes. This was overcome in drilling due to employment of a twist drill which removes bone debris. In addition, more viable osteocytes were observed around microdrill holes. Drilling which was accompanied by cooled irrigation does not produce heat necrosis maintaining viability of adjacent bone. In the same study, when the drilling was performed to 6 mm compared to 2 mm, greater subchondral hematoma was observed with increased access to marrow stroma [21]. In addition, deeper drilling also enhances repair and remodeling of subchondral bone in a rabbit model. Increased bone activity and remodeling achieved through deep drilling improves the cartilage resurfacing. Since the volume of subchondral bone participating in the repair increases as a result of deeper drilling, the quality as well as quantity of cartilage repair is improved [194]. This was confirmed in another study where the quality of cartilage repair as a result of deeper drilling was investigated in a rabbit model. The outcome of cartilage repair indicated by %fill, %Saf-O, %Col-I and %Col-II was significantly better than shallow drilling [17].

The biological response induced by BMS is divided into 3 phases-necrosis, inflammation and repair [195]. Necrosis characterized by varying levels of tissue death begins immediately after injury. In the next phase of inflammation, a dense fibrin network is formed by transudation and cellular exudation mediated by reactive hyperemia and increased capillary permeability. Finally, the repair begins by invasion of fibrous tissue with neovascularisation followed by formation of granulation tissue. In a complex cascade of events, the granulation tissue remodels into the cartilage repair tissue. The repair in marrow stimulated procedures begins with formation of a blood clot in the drill holes and the lesion as a result of subchondral bleeding. The wound repair is initiated by formation of a vascularized granulation tissue accompanied by infiltration of leukocytes. This phase is also characterized by migration of mesenchymal progenitor cells derived from subchondral bone into the defect and increase in their proliferation. The cells then commit to a chondrogenic lineage as condensation of mesenchymal progenitor cells is initiated under influence of PTH/PTHrP. Cell-cell interactions are further promoted by N-cadherin promoting chondrogenesis [18, 192, 196-198]. Chondrogenic foci are detected at this stage, mostly present adjacent to the edges of drill holes, in close proximity to the intact bone. The hyaline cartilage is formed by appositional growth of these foci [18, 196]. Col-I is present in abundance throughout the new repair tissue in the beginning. The matrix gradually witnesses a shift in expression of collagen from type I to type II since the expression of Sox-9 upregulates Col2a1 gene. This is accompanied by rounding of chondrocytes. The organization and constitution of matrix gradually begins to resemble the natural cartilage and there is an increase in the deposition of GAGs and col-II which are abundant in the intermediate and deeper zones of the repair tissue. The col-I content is gradually reduced to few layers on the surface. The chondrocytes in the basal zone undergo hypertrophy induced by expression of Runx2 in later stages of repair. While Sox-9 is a master regulator of chondrogenesis, Runx2 regulates terminal differentiation of chondrocytes and osteoblast differentiation [199]. The foci in the deeper zone are resorbed, accompanied by increased vascularization and bone remodeling bringing about the repair of subchondral bone restoring its porosity and continuity [196, 198].

The origin and differentiation of cells during a BMS repair were outlined in rabbit model by Shapiro *et al* [14]. Fibrinous arcades were seen in the first few days with mesenchymal cell in

growth oriented along long axes. At day 10, proteoglycan content appeared in the matrix followed by presence of a fibrocartilagenous tissue by 2 weeks. The cells progressively differentiated into chondroblasts, chondrocytes, and osteoblasts at week 6, 8 and 10 respectively. Cartilage and bony matrices were formed by week 12 and the regeneration was completed by establishment of tidemark and subchondral bone by week 24. ^{3}H-Thymidine and ^{3}H-cytidine labelling showed that the cartilage repair was entirely mediated by subchondral mesenchymal cells and not from the residual adjacent cartilage [196].

The repair tissue formed after BMS could be a cartilaginous tissue with chondrocyte like cells and cartilaginous matrix. In other cases, a non-cartilaginous tissue is formed which might be fibrous, intermediate or chondroid with increasing restoration of cartilaginous phenotype [192]. On the examination of structure of early repair tissue in BMS, cartilage appears as focal plaques instead of a continuous layer. In most of the cases the repair tissue has a streaky, fibrous appearance. The arrangement of cells is random with a zone of flattened cells just below the surface resembling the natural cartilage. Calcified cartilage and tidemark are not clearly distinct. Although the proportion of repair tissue increases with time, the results are variable and influenced by age and species of the animal in addition to location and size of lesion [192]. Young animal defects are healed better than old probably due to higher number of mesenchymal progenitor cells with superior differentiation potential [192]. In addition, smaller defects show superior healing in the initial stages but suffer from rapid breakdown later on due to poor subchondral bone healing [200]. In other animals, poor repair was observed in weight bearing, large defects despite a good healing in the beginning. Weight bearing, large defects are more likely to be affected by opposing articular surfaces which impinge on the fragile repair tissue leading to its poor attachment [199].

In a systematic review of existing clinical data, Mithoefer *et al* reported that MFX provides effective short term functional improvement and patient relief [201]. The improvement was observed as early as six months and improved consistently for a period up to 24 months. Limited hyaline repair tissue, variable repair cartilage volume and possible functional deterioration are some of the shortcomings of the procedure. Younger patients had better clinical outcome scores which was a result of better cartilage repair fill. Medial femoral condyle was found to repair

poorly in most of the studies. Most importantly, MFX is mostly recommended as a first line of treatment for defect sizes <4cm^2 [201].

Some of the primary causes of failure of MFX include clot retraction and presence of calcified cartilage at the base of defect [199, 202]. Age, location and size of defects are critical parameters to be considered while proceeding with MFX [199]. Poor subchondral bone repair, necrosis of adjacent articular cartilage and advancing of repairing subchondral bone are still other shortcomings that have been reported [203]. One of the reasons of failure of BMS is the poor integration of a well repaired tissue with the surrounding tissue leading to early degeneration [14]. Clefts that develop between reparative tissue and subchondral bone are a common cause of failure of marrow stimulated repair in long term [199]. Another probable cause of the failure of marrow stimulated repair was shown to be the presence of hypocellular adjacent matrix with poor restoration of matrix components, primarily its proteoglycan content. The incomplete matrix repair fails to support collagen fibres [199]. In addition to this phenomenon, BMS fails in weight bearing regions or larger lesions due to incomplete bone remodelling. The subchondral bone fails to support the newly formed cartilage tissue which needs to rely on still underdeveloped matrix proteoglycans [199]. Presence of calcified cartilage (CC) layer was shown to be detrimental to the repair and regeneration of cartilage repair tissue by Hurtig *et al* [199]. It acts as a barrier to invasion of fibrous tissue from subchondral bone. Frisbie *et al* carried out a detailed study to examine the effects of calcified cartilage on repair outcome and concluded that amount and attachment of repair tissue was enhanced upon removal of CC while retaining subchondral bone [204]. The remodelling of subchondral bone was also improved upon removal of CC by enchancing the diffusion of nutrients from subchondral bone [188].

2.5 Influence of ageing on proliferation and differentiation potential of BMSCs

BMSC ageing has been described as irreversible arrest of cell division and expression of senescence associated β-galactosidase [105, 205, 206]. Older BMSCs usually undergo senescence which is different from apoptotic cells in that they are alive but cannot undergo anymore divisions due to irreversible G1 growth arrest [207]. Moreover, young cultures comprise more small, spindle shaped cells while there are more flattened, broad, slow dividing cells in

older cultures (Fig. 2.10) [208, 209]. This phenomenon was first coined the Hayflick limit by Leonard Hayflick in 1960s. Aged BMSCs show a decline in maximal life span and growth rate [210, 211]. In addition to altered morphology, senescent BMSCs display upregulated levels of p53/p21 and p16/Rb cell cycle inhibitors. In general, ageing induced changes in BMSCs can be broadly classified as: 1). Change in quantity, 2). Change in quality indicated by differentiation capacity, 3). Altered mobility [212]. BMSCs displaying senescence are characterized by limited differentiation potential, limited self-renewal capability, limited by Hayflick limit, and inactivated telomerase [205]. One of the most important factors affecting the number, proliferation and differentiation potential of BMSCs is ageing. While fetal bone marrow contains 1 in 10,000 BMSCs, the number reduces to 1 in 250,000 in adults [213]. Since older BMSCs still remain metabolically active, senescence and not apoptosis is believed to be the primary cause of reduction in proliferation capacity [111]. Quarto *et al* in 1995 pointed out that CFU-f was inversely correlated to age leading to decrease in differentiation potential in senescent animals [214]. Muruglia *et al* provided evidence that frequency of tripotential clones reduces with increasing age replaced gradually first by bipotential and then unipotential clones thus reducing the differentiation potential [100]. In addition, the ability of cells to migrate in response to chemoattractants also reduced with increasing age. This may have a profound influence on cartilage repair since migration and recruitment of BMSCs will be reduced leading to poorer or slower repair in older individuals.

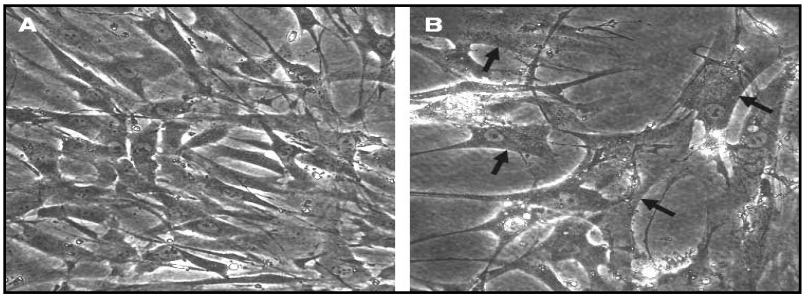

Figure 2.10: Cellular morphology of (A) early and (B) senescent BMSCs. (A). Cells are narrow and spindle shaped in younger passage. (B). Large and flattened senescent BMSCs in late passage are shown by arrows. [*Image copied from Wagner et al*-215].

In an interesting observation by Stenderup *et al*, human BMSCs were found to undergo lower number of population doublings before entering senescent phase compared to other species [210]. It is estimated that stem cells lose 17bp/yr and stop dividing when telomere length is reduced to 10 kbp, thus reducing the proliferation in older individuals. Interestingly, according to some reports, MSCs possess very low levels of telomerase, the enzyme responsible for maintaining telomere length [216]. In fact, telomerase activity is not detected in huMSCs [217]. In addition, there is a downregulation of genes involved in DNA replication, cell cycle, mitosis and DNA repairs at older age [218]. Simonsen *et al* showed that overexpression of telomerase in MSCs abolished senescent phenotype and maintained their capacity to proliferate and differentiate [216]. Moreover, Liu *et al* showed that MSC without telomerase activity lacked the capability to differentiate into adipocytes and chondrocytes [219]. These studies suggest that telomerase is critical for cell replication and differentiation [105].

In their study, Payne *et al* did not observe correlation between age and proliferation, however, the chondrogenic potential was reduced which was reflected by reduced sizes of pellets initiated from cells obtained from older individuals [220]. In yet another study, while there was poor expression of Col-II, GAGs were not deposited at all in old rats [221]. Moreover, the expression levels for mRNA for Col-II, aggrecan and link proteins were lowest in old animals. In the classic study of Meachim and Roberts, it was reported that the cartilage repair outcome improves with increasing time in an animal model and was better in younger animals [192]. Finally, younger patients were found to show better repair outcome in clinical studies comparing MFX and ACI, conducted by Knutsen *et al* and Saris *et al* [222-225]. It can thus be concluded that BMSCs lose proliferation as well as differentiation potential with increasing age which is expected to influence the cartilage repair outcome in marrow stimulated procedures.

A significant decrease in the growth rate has been observed in BMSCs derived from aged donors [211, 226]. Moreover, older BMSCs also failed to show an increase in proliferation upon positive stimulation unlike young BMSCs [211]. Li *et al* showed a decrease in proliferation of BMSCs with increasing age and correlated it to decrease in the clonogenic potential [227]. Correlation between age and CFU is controversial since some studies have observed an effect of age on CFU-f [211, 228-230] while others indicate CFU is unaffected by age of donor [231-233]. In another study, BMSCs from older donors displayed a reduced initial proliferation phase compared to

younger BMSCs [234]. Interestingly, Bustos *et al* reported a significant impairment in the migration potential of BMSCs derived from older donors [235]. Similar results were also reported by Naaldijk *et al* who observed a minor reduction in migration potential of BMSCs from old donors [236].

Several groups have worked with *in vitro* cultures of BMSCs derived from different species and reported that ageing influences multiple biological processes related to the BMSC function including: 1). Reduced yield of BMSCs in BM aspirate, 2). Reduced proliferation and differentiation potential, 3). Reduced CFU-f frequency, 4). Increase in frequency of senescent population, 5). Modification in morphology of BMSCs from spindle shape to flattened, 6). Altered expression of cell surface markers and finally, 7). miRNA expression and immunomodulatory potential [237-240]. Moreover, the therapeutic potential of BMSCs was found to be influenced in an age-dependent manner in recent *in vivo* studies as well including increased susceptibility to reactive oxygen species, altered immunomodulation and impaired angiogenesis [241-243]. Fehrer *et al* proposed influence of ageing to be similar to heirarchial model of lineage [105] since a progressive loss of clonal multipotentiality of BMSCs is observed in senescent BMSCs. Studies have also reported a reduction in the proportion of RS cells and CFU-f in the populations derived from older donors. Colony size of CFU-fs also reduced in BMSCs derived from older donors [244]. D'Ippolito proposed that aging of BMSCs is accompanied with loss in proliferation and multipotential differentiation [245].

Although telomere shortening is considered to be one of the most important factors contributing to age-dependent modified behaviour of BMSCs, other studies report several pathways involved in senescence. DNA damage, accumulation of cyclin-dependent kinase inhibitor p16INK4a and oxidative stress have been shown to have detrimental effect on properties of ageing BMSCs [246-248]. Moreover, downregulation of genes involved in cell cycle, DNA replication, mitosis and DNA repair have been reported in higher passages indicating older BMSCs may have altered DNA repair and regulation mechanisms [215]. Stolzing *et al* reported an increase in nitric oxide, ROS, oxidized and glycated proteins levels in addition to reduction in superoxide dismutase activity in older patients [209]. Oxidative stress induced apoptosis and inhibition of differentiation was more pronounced in older BMSCs [209]. Other studies have shown oxidative damage to lead to BMSC apoptosis and senescence [249, 250] and inhibition of differentiation

[251]. Increased level of p53 levels has been associated with increasing age and inhibition of p53 has been shown to have a positive impact on osteoblastic differentiation [252]. P53 mediated senescence is attributed to telomere dysfunction and DNA damage while p16 /RB mediated senescence pathway is impacted by oncogenes, chromatin disruption and stresses [206]. Ageing has also been shown to impair the HSP/stress response accompanied by reduced differentiation and proliferation [253]. In addition, the expression of cell surface markers such as CD105 and Stro-1 has been shown to be altered in older BMSCs [231]. Older BMSCs have also been shown to have reduced expression of TGF-beta and BMP 2/4 [254]. Reports suggest that ageing induces changes in composition and immunophenotype of BM which in turn may alter functional behaviour of BMSCs during development and post-natal life [58]. *In vivo*, increasing age causes increased cross-linking of collagen. Advanced glycation end (AGE) products inhibit *in vitro* adhesion and spreading of BMSCs on collagen [255]. Moreover, AGE modified BSA induces apoptosis while inhibiting proliferation and differentiation [256]. This observation is underlined by the fact that BMSCs isolated from fetuses and pediatric donors have higher proliferation potential and lesser population doubling time compared to adult BMSCs (Fig. 2.11) [257, 258].

The detrimental effect of ageing is mimicked in older long term cultures of BMSCs and reduced differentiation potential was observed in long term cultures [211, 259-261]. Murine and rat BMSCs have been reported to demonstrate karyotopic aberrations and malignant transformations upon long term culture [262-264]. Osteogenic potential was also reported to be diminished in older donors across several species [205]. In fact studies have shown that similar genes are upregulated in old long term cultures and older people [89, 265].

Reports suggest epigenetic mechanisms as underlying causes of ageing dependent alterations in BMSCs [266]. DNA methylation is one of the most important epigenetic mechanisms studied in mammals. Several osteogenesis related markers have been shown to be influenced by DNA methylation patterns [267, 268]. Histone modifications have also been implicated in determining the functional behaviour of BMSCs. Histone modification pattern has been shown to be constantly modified during osteogenesis. Age related impact on these epigenetic mechanisms may have profound bearing on downstream differentiation of BMSCs. Yang *et al* implicated JNK pathway mediated reduction in the migration ability of BMSCs from aged donors [269].

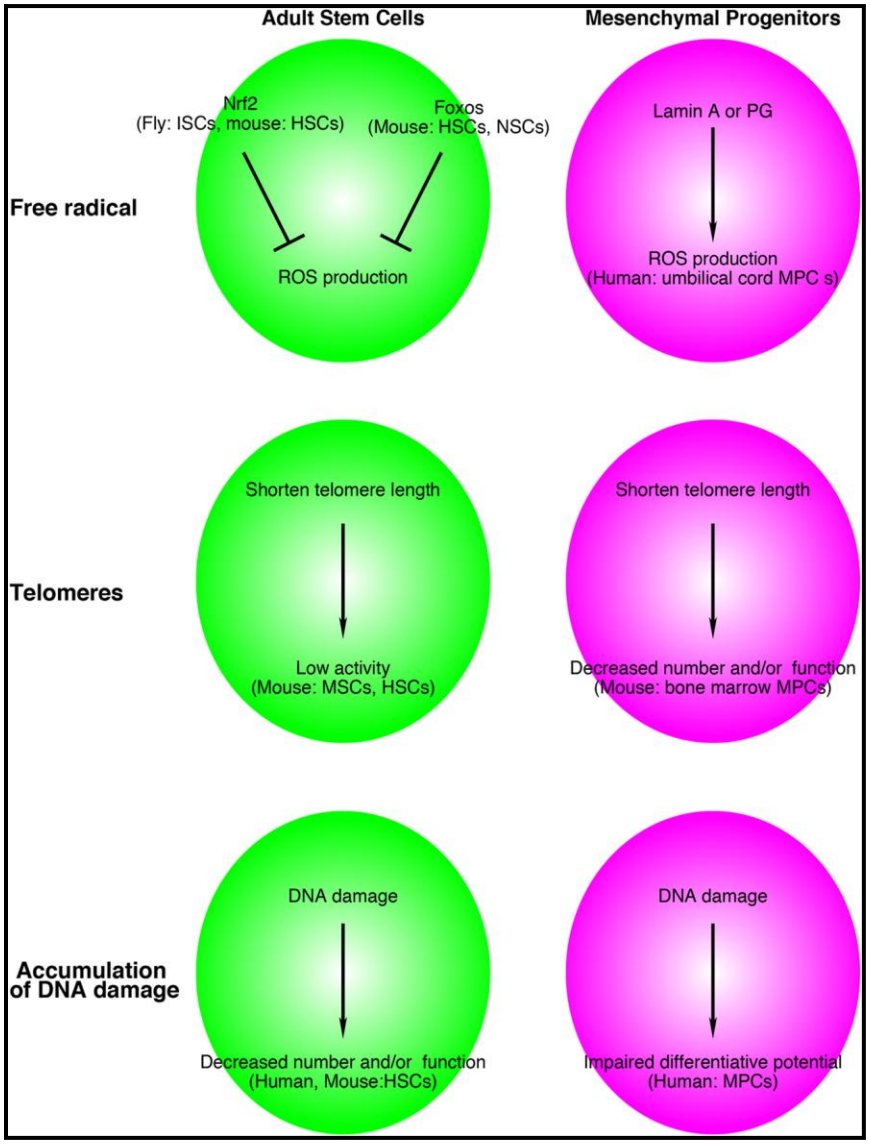

Figure.2.11: Figure illustrating the relationship between adult stem cell and mesenchymal progenitor theories of aging and free radicals, telomeres, and accumulation of DNA damage. [*Image copied from Fukada et al*-270].

2.6 Influence of location of defect on the marrow stimulated cartilage repair outcome: Condyles v/s trochlea

In clinical scenario, the medial femoral condyle (MFC) is the most commonly affected area accounting for 34–58% of chondral injuries [6, 271] compared to 6-8% cases where defects are located on trochlea. The pre-clinical and clinical studies reporting the influence of location of defect offer conflicting scenarios [272, 273]. While Steadman reported that there was no

association between location and cartilage repair [274]; other studies reported superior clinical scores in repair outcome of condyles [275, 276]. An ovine study conducted by our group also highlighted difference in the repair outcome between trochlea and condyle. Condyle demonstrated a higher %fill, better matrix restoration and subchondral bone healing. This indicates the existence of species specific factors which affect the repair outcome based on location of defect [13]. When deep drilling was employed for cartilage repair in a rabbit defect model, improved repair outcome was observed in trochlea compared to shallow drilling possibly because deep drilling provides access to metaphyseal marrow. However, this observation was not consistent in condyle. At the end of three weeks, volume density of chondrocytes and number as well as area of chondrogenic foci was higher in rabbit trochlea [12]. It is known by now that chondrogenic foci which arise from subchondral bone progenitors mediate the cartilaginous repair by transforming into cartilage repair tissue bringing about marrow stimulated cartilage repair in animal defect model [18]. Trochlea had more mature foci while nascent foci were observed in condyle although the volume fraction of marrow derived progenitor cells in surgical holes was similar in both cases. Moreover, woven bone formation was faster in condyle, but was comparable to trochlea in later stages [11, 196]. The observations were confirmed in a longer three month study where poorer repair outcome was observed in condyle. While Col-II expression in repair tissue was comparable, %fill was significantly lower in condyle accompanied by higher % Col-I indicating more fibrocartilagenous repair. Moreover, the subchondral bone density was completely restored in trochlea while condyle had lower subchondral bone fraction. Interestingly, even the shallow trochlear defects demonstrated superior repair than condyle indicating that the inability to access metaphyseal marrow in the case of condyle is not the only cause for poor repair in latter [11, 12].

This difference in the repair outcome between condyle and trochlea could arise due to structural differences such as thicker cartilage and subchondral bone plate along with higher subchondral bone density observed with condyle which might reduce the recruitment of the progenitor cells from the subchondral bone [277]. Several intrinsic differences are observed between condyle and trochlea-the convex v/s concave surface anatomy, load-bearing features, biomechanical cues and biological factors including chondrogenic potential of marrow-derived precursors [223, 278]. Biomechanical cues such as dynamic compression is believed to improve chondrogenic

differentiation [279-281]. Rabbit condyle is weight bearing while trochlea is partially loaded. The concave surface of trochlea might shield the fibrin clot and granulation tissue from the shear forces to which condylar defects are continually exposed due to convex surface of the latter [19, 196]. Finally, there could be the differences in the inherent properties of progenitor cells present at these locations which might be a function of age or cellularity influencing the number as well as differentiation potential. The differences in the biological properties of trochlea and condyle might arise as a result of intrinsic or developmentally derived biological properties or different load-bearing patterns.

Moreover, the regenerative potential of MSCs has been attributed to their paracrine effect. Cytokines, adhesion molecules and growth factors play a critical role in healing role of MSCs [82]. It becomes imperative to wonder if it is likely that the reduced progenitor population in condyle might lead to poor regeneration of repair tissue. In addition, angiogenins expressed by stromal cells induce processes essential for bone growth including capillary proliferation, expansion of the sinusoidal space as well as vessel growth and remodeling, processes that are all essential for bone growth [282]. Thus, a lower yield of condylar BMSCs may also lead to poorer bone remodelling-an important aspect of osteochondral repair.

Since methods for *in vivo* localization of BMSCs still elude us, it becomes imperative to utilize the *in vitro* assays to shed light on the underlying mechanisms of differences observed in cellular and functional behaviour of BMSCs. For example, donor age and disease stage have been shown to impact the proliferative and differentiation potential of BMSCs in several studies [283]. Moreover, BMSCs obtained from OA patients have been shown to have reduced potential for proliferation and chondrogenesis compared to healthy BMSCs [53].

Bone marrow stimulation procedures rely heavily on the migration of BMSCs to the repair site. Although the mechanism of BMSC migration is still uncertain, many believe that chemokines and adhesion molecules play an important role in migratory behaviour of BMSCs [284]. For example, the chemokine receptor type 4 (CXCR4) and its binding protein stromal-derived factor 1-α (SDF-1α) are essential role for the process [285]. An inherent difference in the migration potential of BMSCs arising due to differences in receptor profile may also lead to variability in cartilage repair outcome.

Hierarchichal structure of BMSC differentiation has been proposed [286]. Russell *et al* showed that tri-potent clones had a higher rate of proliferation and reduced apoptosis [127]. Currently, markers to distinguish multipotent BMSCs from more committed progenitor cells are not available. As a result, a difference in the lineage commitment profile of BMSCs is hard to ascertain. A difference in the lineage commitment of BMSCs in condyle and trochlea may impact their potential for proliferation and differentiation-both with a profound impact on cartilage repair potential.

Studies have reported that histone modification patterns on BMSCs can be correlated to its multipotential state and eventually to the differentiation potential. It will be interesting to explore if there are any differences in the epigenetic mechanisms of regulation between condyles and trochlea [287]. In an important study it was reported that BMSCs obtained from different organs had very similar profiles with minor differences in differentiation potential and surface marker profile. Interestingly, it was proposed that this difference may arise due to influence of local environment of origin and may be niche-dependent [119]. The origin or location of BMSCs' source therefore has a bearing on their properties.

2.7 Application of chitosan in improving cartilage repair

Platelets and chitosan were first decribed by G. Bizzozereo and C. Rouget respectively [288]. BMS procedures rely on the formation of a blood clot which is retracted to a fraction of its size due to clot retraction property of platelets and exudation of serum. The contraction leads to shrinkage of clot and detachment from lesion surface. Platelets are the central hemostatic component comprised of anuclear, discoid shaped cell and are rich in several growth factors including TGF-beta, PDGF, VEGF etc in addition to cytokines [289]. Exposure to collagenous subendothelial structures causes platelets to adhere and bind with each other. The aggregated plug retracts and releases components which in turn induce inflammation and wound repair [290]. The retraction of the clot is mediated by actin-myosin contractile apparatus in the platelets and leads to loss of more than half of the clot's original volume through serum exudation [291]. This retraction is detrimental for cartilage repair since it leads of shrinkage of clot and ultimate detachment of repair tissue from the defect bed [13, 14, 19, 292].

Chitosan is a biopolymer derived by deacetylation of chitin obtained from shell of crustaceans [293]. It is a linear polysaccharide comprised of β (1-4) linked D-glucosamine and various groups of randomly located N-acetyl glucosamine residues (Fig. 2.12). The average weight of CS is dependent on the method of preparation and can range from 50 to 1,000 kDA. The degree of deacetylation is generally in the range of 50-90% [294].

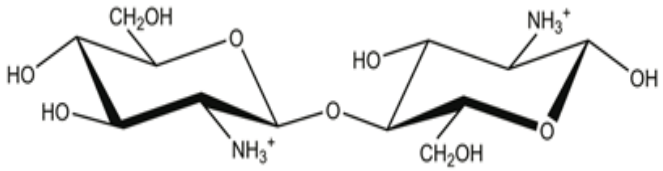

Figure 2.12: Schematic of structure of chitosan- cationic polymer of partly acetylated glucosamine, made up of β (1-4) linked D-glucosamine and various groups of randomly located N-acetyl glucosamine residues. [*Image copied from Buschmann et al*-295].

Its beneficial role in repair of skin, cornea and bone among other tissues has been shown in past [296-298]. CS is an ideal scaffold material since it is biocompatible, soluble at physiological pH and is resorbed in a reasonable time frame [295]. Moreover, it inhibits platelet mediated clot retraction while promoting coagulation due to its hemostatic properties attributed to its ability to chain erythrocytes and activate platelets (Fig. 2.13) [299-301]. Finally, due to polycationic nature and high charge density, CS has excellent adherence to negatively charged biological tissues including bone and cartilage.

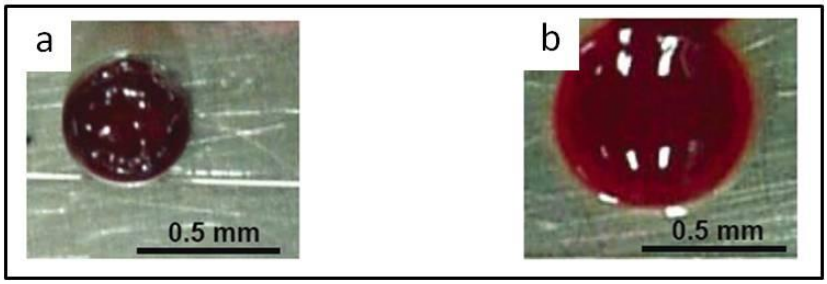

Figure 2.13: Chitosan inhibits the platelet mediated retraction of blood clot leading to negligible loss in volume (b) compared to > 50% loss in volume in clot formed by blood alone (a). [*Image copied from Buschmann et al*-295]

Chitosan has been shown to be extremely biocompatible in several studies. It invokes a minimal foreign body reaction with negligible fibrous encapsulation [302]. The beneficial role of chitosan with respect to tissue engineering and regeneration includes its ability to encourage healthy granulation tissue formation, accelerated angiogenesis, increased cell proliferation and improved integration of repair tissue with host tissue [294]. In addition CS has unique hemostatic properties and modulates the migration behaviour of immunomodulatory cells such as neutrophils and macrophages [303, 304]. The beneficial effects of CS can be briefly summarized to include the following-chemoattraction, enhancement of granulation tissue formation, restricted retraction, hemostatic property, activation of immunomodulatory cells including neutrophils and macrophages and promotion of ECM synthesis by release of glucosamine and N-acetylglucosamine residues [305].

Chitosan oligosaccharides have a stimulatory effect on macrophages due to acetyl residues and CS has been shown to exert chemoattractive influence on neutrophils *in vitro* as well as *in vivo* [306-308]. *In vivo*, CS induces a significant recruitment of neutrophils in its vicinity without developing a chronic inflammatory response [294]. This property likely attributes to increased local cell proliferation and better attachment of repair tissue.

Owing to these properties, CS has been developed as an ideal scaffold material for tissue engineering application among others. In addition, N-acetyl moiety in CS has structural similarities with GAG and HA component of cartilage matrix and makes it application for cartilage TE even more ideal [294]. The structural similarity with GAGs increases its potential for bioactivities related to interaction with growth factors receptors and adhesion proteins [294]. Repair of cartilage lesions was improved when chitosan was used along with BMP-7 in a rabbit cartilage defect model [309]. Chitosan-HA hybrid showed significant improvement in chondrocyte adhesion, proliferation and synthesis of cartilage matrix proteins [310]. Moreover, CS based scaffolds ensure a controlled release of growth factors which promote cartilage repair by increasing chondrocyte proliferation, recruitment and biosynthetic activity [302]. Collagen/CS/GAG scaffold developed by Lee *et al* ensured a controlled release of TGF-βI and promoted cartilage regeneration *in vitro* [311]. CS scaffolds maintained the rounded morphology of chondrocytes along with synthesis of cell-specific ECM molecules [312, 313]. When used with PLLA films, CS improved cell adhesion, proliferation and biosynthetic activity of

chondrocytes [314]. Along with chondrocyte proliferation, CS/HA scaffold improved the synthesis of collagen type II and aggrecan [310]. CS/HA/alginate scaffolds developed by Hsu *et al* showed neocartilage formation *in vitro* and improved repair upon transplantation in rabbit knee [315].

In addition, CS scaffolds have also been used with GFs alone for improved regeneration. PDGF and FGF encapsulated within CS promoted the differentiation of adipose derived stem cells (ADSC) and GAG synthesis. Similarly, controlled release of GFs such as IGF-1 and BMP-2 from CS porous scaffolds improved bone regeneration *in vivo* [316]. Encapsulated VEGF and PDGF improved revascularization in a rabbit model [317] . CS scaffolds promoted chondrogenic differentiation of ADSC indicated by significant improvement in GAG and collagen type II deposition along with upregulation of cartilage markers including Sox-9 [318]. Osteogenic differentiation of ADSC was promoted by platelet lysate released from CS-Chondroitin sulfate nanoparticles indicated by increased mineralization [319]. Defects in cranial bone were repaired in 16 weeks by PRP-$Ca_3P_2O_8$-CS scaffolds by newly formed lamellar and woven bone due to the beneficial effect of GFs released from the scaffold on the BMSC differentiation [320, 321]. BMSC differentiation into chondrocytes was shown in a thermosensitive water soluble CS poly (N-isopropylacrylamide) gel with ability to be injected into the site of defect in a liquid form followed by its solidification at physiological temperatures [322].

The causes for the better repair observed with CS-GP/blood implants were investigated in a rabbit model. Chitosan clots improve the attachment of the repair tissue growing from marrow holes, since only as little as 1% of tissue was detached in case of treatment. In addition, chitosan clots increase the cell recruitment into drill holes resulting in elevated levels of progenitor cells in the lesion. The transient subchondral angiogenesis is promoted which results in increased vascularization including large blood vessels in deeper zones and small vessels in superficial zone leading to a porous and revascularized bone plate [19, 291]. Improved subchondral bone remodeling allows higher tissue regeneration and better attachment of repair tissue [19]. Interestingly, chitosan improves the bone remodeling and repair even when residual calcified cartilage layer is present [19]. Chitosan clots release IL-8 which increases neutrophil and bone marrow progenitor cell recruitment in addition to stimulating angiogenesis. Increased recruitment of neutrophils in turn escalates the infiltration of alternatively activated macrophages which

improve subchondral bone repair due to their pro-angiogenic property [323]. Moreover, the levels of osteoclasts were increased by 2-fold which led to better subchondral bone remodeling and superior bone repair [20]. Our group also observed that osteochondral repair is mediated by formation of chondrogenic foci which recapitulate MSC condensation and chondrogenesis in embryonic limb bud. Although delayed by treatment with chitosan-GP/blood implants, the number and area of mature foci was higher. Moreover, the foci were displaced towards articular surface leading to reduced resorption to bone [18]. Quality as well as quantity of repair tissue in lesions treated with MFX and CS-GP/blood implants indicated a superior outcome in ovine models at the end of 6 month period. In addition, the incidence as well as severity of subchondral cysts-a common occurrence in ACI as well as BMS procedure- was reduced in this model [13].

In a study including 33 patients on a compassionate basis, chitosan-GP/blood implant (BST-Cargel) was proven to be safe for human application. Moreover, 12 month WOMAC (Western Ontario and McMaster University Osteoarthritis Index) scores showed considerable improvements in pain, stiffness and function over the baseline scores [324]. In summary, the application of a chitosan-GP/blood implant increases the quality and quantity of cartilage repair tissue which integrates better with a porous and healthy subchondral bone compared to BMS alone. Stanish *et al* published the results of a 1 year randomized controlled trial comparing BST-CarGel treatment with MFX alone in 80 patients. Lesion fill and repair quality were improved in the BST-CarGel group (41 patients) compared to MFX alone (39 patients) indicated by more hyaline cartilage like T2 values [325]. Osteochondral biopsies showed significant improvement in ICRS macroscopic score in BST-CarGel group compared to MFX alone indicating improvement in fill, integration and appearance of tissue. In addition, significant improvement was also observed in surface architecture, surface/superficial assessment and cellular parameters in BST-CarGel treated defects. Collagen stratification was improved and resembled native cartilage more closely in the BST-CarGel group. The MFX group did not show a significant improvement in any of the parameters studied indicating superiority of BST-CarGel treatement over MFX alone [326]. The 5 year results from the study showed significant improvement in lesion filling and T2relaxation times with BST-CarGel versus MFX alone. Although the clinical benefit by both the procedures was same, BST-CarGEl induced more sustained and significantly superior quality and quantity of repair tissue [327]. The technology was acquired by Smith and

Nephew and is currently available in 26 countries. Recognizing the need for a one-step cell based method, BST-Cargel was subsequently combined with bone marrow aspirate concentrate in order to utilize the cartilage regeneration potential of MSCs to treat challenging defects. *In vitro* properties of the product - including clot retraction, cell viability and morphology, clotting time and product degradation - were reported to be similar to the currently available BST–CarGel implant [328]. Steinwachs *et al* and Al-Qarni *et al* recently published reports on the arthroscopic use of the technique [329, 330]. Although minimally invasive, the arthroscopic technique is technically challenging and not suitable for lesions in certain locations.

2.8 Application of PRP in improving cartilage repair outcome

A possible means to further improve the above strategy of BST-CarGel product is by using platelet-rich plasma (PRP) instead of whole blood. The use of PRP for clinical application was suggested by Marx *et al* in 1998 [22]. PRP is prepared by sequential centrifugation of whole blood and is defined as a volume of the plasma fraction of autologous blood having a platelet concentration 4-6 times the baseline [22-24, 331]. The platelet concentrate is activated following the separation from erythrocyte supernatant using either calcium chloride or thrombin. As a result of activation, PRP's secretome - which may contain up to 800 protein components- is released [332]. In addition to growth factors, PRP is primarily composed of soluble mediators involved in inflammatory response-such as IL-1RA, IL-4, IL-8, IL-10, TNF-α, α-2-macroglobulin and mediators with a modulatory role in angiogenesis and coagulation [333]. PRP preparations are highly heterogeneous arising from discrepancies related to starting number of platelets, use of anticoagulants, isolation method, presence of leukocytes and use of activators [334-337]. A method for classification of PRP has been developed in order to address the high variability in cell composition of PRP. The international classification system-PAW classifies PRP based on the following criteria: absolute platelet count-low (P1) to high (P4), manner of platelet activation and presence or absence of white blood cells [338]. Based on the method of preparation, PRP may contain cells from leukocytic lineage, and is known as L-PRP.

PRP finds application in tissue engineering mainly due to being a rich source of anabolic growth factors and ease of availability of autologous PRP preparation. However, activated platelets tend to release GFs quickly leading to loss of activity and reduced clinical efficiency [305]. Moreover

hyperconcentration of platelets in PRP preparations aggravates the platelet activation leading to rapid reduction or loss in platelet function.

The exact mechanism of PRP in cartilage repair is still poorly understood due to large number of mediators and complex interactions [339] and is likely attributed to the growth factors stored in α-granules of platelets [340]. PRP is safe for clinical application and no medical contraindications have been recognized as yet [340]. Autologous PRP is easy to prepare and easily available and reduces the risk for transmission of disease or rejection of graft [334]. PRP is easy to deliver and is a suitable candidate to increase the GFs concentration in the defect milieu to achieve more successful cartilage repair results. Moreover, the mitotic effect of PRP was found to be reversible following the withdrawal of PRP. This is important since the cells need to retain their normal rate of proliferation once the tissue is regenerated [341].

In an important study, Kutlu *et al* showed that CS was a suitable carrier for PRP application and ensured a sustained release of GFs [342]. Platelet aggregation was shown to be enhanced on a CS/PCL scaffold [343]. Porous freeze dried CS scaffold used for sustained release of TGF-βI from microspheres increased chondrocyte proliferation and ECM synthesis [344]. In another study by our group, it was found that chitosan coats blood-derived cells and fibers in CS/PRP clots and prevents clot retraction through physical inhibition of platelet aggregation. Moreover, the release of EGF and PDGF-AB is increased *in vitro* from CS/PRP clots compared to PRP clots. Based on these studies, CS seems to be an ideal candidate for development of medium which ensures a controlled release of GFs over an extended period of time thereby improving the repair and regeneration outcome.

Studies have shown that growth factors such as TGF-β [345], BMP-7 [346], FGF-2 [347] encourage proliferation and chondrogenic differentiation of BMSCs. It is likely that application of these factors improve the BMS repair outcome. Studies have reported an increase in proliferation and ECM synthesis upon exposure to PRP. The beneficial effects of PRP likely arise from its capability to promote angiogenesis, recruitment of cells to site of defect and induce local GF production [340]. PRP also has a positive impact on bone regeneration since BMSCs contain receptors for growth factors released from PRP which enhance the rate of bone formation and bone density [22]. Lucarelli *et al* observed an increase in the proliferation of MSCs upon exposure to PRP [341]. Properties such as increased BMSC recruitment and angiogenesis can

find an application in cartilage repair to augment the marrow stimulated procedures [348, 349]. PRP also releases Nf-κB pathway mediators such as IGF and HGF thereby inhibiting the IL-1β mediated inflammatory reaction [333, 350-352]. Several groups have used PRP to augment BMS with mixed outcome. An increase in proliferation and anabolic activity of chondrocytes as a result of culture in medium containing 10% PRP was observed by Akeda *et al* [353]. In addition, PRP also has beneficial effects on proliferation and viability of chondrocytes and BMSCs [133-135]. Kruger *et al* pointed out that migration and chondrogenic potential of subchondral BMSCs was enhanced in presence of PRP [26]. Moreover, PRP inhibits the catabolic effects of IL-1β, TNF-α, NF-κB on chondrocytes in *in-vitro* conditions [354, 355]. *In-vivo*, PRP reduces inflammation related Col-II loss and synovial hypertrophy [352]. Finally, PRP is likely to exert a positive effect on the recruitment of BMSCs from the bone to the site of defect due to abundance of chemokines present such as CCL3, CCL 5, CXCL1, CXCL 12 and CCL 17 [356]. An autologous bone graft combined with PRP and MSCs accelerated subchondral bone remodeling in an animal model improving the subchondral bone healing [357, 358]. Huh *et al* showed that PRP improved the outcome of BMS compared to BMS alone in a rabbit model [340]. However, they did not evaluate the mechanical strength of their repair tissue vis-à-vis the natural cartilage. PRP was also found to enhance the chondrogenic differentiation of BMSCs [359]. In a study with 14 patients, PRP injections provided relief in OA patients at 12 month follow up [360]. In their systematic review, Smyth *et al* concluded from 21 *in vitro* and *in vivo* studies that PRP increased the synthetic capacity of chondrocytes along with increased proteoglycan and collagen synthesis thus increasing the hyaline content of the repair tissue [25].

On the contrary, studies carried out in human, bovine and ovine cells have indicated that while PRP may enhance the proliferation, it has deleterious or no effect on the chondrogenic differentiation [361-363]. The downside of using PRP derives from the sudden burst release of GFs post activation of platelets. The platelets release the contents of α-granules within 10 minutes of activation and release over 90% of their contents within 1 hour. Owing to the burst release of GFs, some authors have questioned whether it is possible to regenerate cartilage defect upon such a short exposure to GFs [24, 364]. The effect of PRP on the chondrogenic differentiation has been controversial where some studies found an increase in the deposition of GAGs and collagen type II [353, 365]. On the other hand, Drengk *et al* showed that increased proliferation of chondrocytes was accompanied by reduction in the chondrogenic phenotype in

the presence of PRP [363]. Similar results were seen by Gaissmaier *et al* who cultured chondrocytes in presence of PRP. Although PRP induced an increase in the proliferation in monolayer phase, the chondrocytes were dedifferentiated into a fibroblast like phenotype in association with downregulation of collagen type II, aggrecan and BMP-2 mRNA levels in addition to significant upregulation in collagen type I [362]. In their study, Li *et al* observed a sustained expression of stemness related markers while expression of lineage specific markers was reduced. While PRP induced proliferation in muscle derived MSCs, it maintained cells in a more undifferentiated state and cells did not show any change in the osteogenic or chondrogenic differentiation capacities [366]. Nasal septal chondrocytes were stimulated for proliferation but failed to redifferentiate in 3D cultures and expression of cartilage specific matrix components was not achieved in the presence of PRP [361]. Using an ovine chronic defect model, Milano *et al* showed that when used as an adjunct to BMS, PRP enhanced the repair response versus BMS alone [367]. Although the application of PRP improved the macroscopic and mechanical outcome, the hyaline nature of RT was lacking.

2.9 Considerations for suitable animal model

In order to relate animal models with human repair and identify a model which not only corresponds to the older and degenerative human joints, but also can be used for development of strategies for better repair, it is crucial to choose the animal model carefully. Since subchondral bone is the source of repair in case of BMS procedure, it becomes important to consider the subchondral bone parameters as well as cartilage properties while choosing an animal model. Rabbit trochlea resembles human MFC more closely than any other species in subchondral bone porosity, thickness of bone plate as well as repair outcome [368].

In this study, biological properties of rabbit BMSCs from condyle and trochlea will be determined. These will then be correlated with the repair outcome in a skeletally mature rabbit model. While developing the animal model, it is important to bear in mind a few considerations which include but are not limited to the following. Young animals have tremendous spontaneous repair potential and are an unsuitable candidate for development of models for cartilage repair studies [13]. Moreover, skeletally mature animals are more comparable to the patient population in need of cartilage repair treatment. The size of the defect is an important consideration. Defects less than 3 mm have been shown to repair spontaneously [199] and therefore, it is important to create critical sized defects to rule out the possibility of spontaneous repair. In the rabbit model developed by our group, 17-20% of defect area is perforated by drill holes by placing 4 holes in a 4X4mm defect in rabbit. This simulates the clinical hole perforation density range of 13-49% used in humans. In addition, the rabbit defect size is approximately 1/10th of average clinical defect size of 1.5 cm^2. As a result a scaling factor of 3 is used for linear dimensions reflected by the ratio of diameter of clinical tool (3 mm) and microdrill to be used in this study (1 mm) [21].
Although rabbit knee has been widely used in experimental cartilage repair studies [194], it is imperative to take certain considerations into account while extrapolation of results. The model proposed in this study is an aggressive model since bilateral defects will be created on trochlea as well as condyle resulting in large surface being covered by defects which might lead to formation of osteophytes [223]. It is important to create bilateral models owing to high interindividual variability observed with rabbit model [194]. Also, rabbits have high degree of flexion as a result of which the load bearing region of MFC is more posterior than human. Bone marrow stimulation procedures rely on the recruitment of progenitor cells from the underlying subchondral bone. Drilling deep has been shown to provide access to metaphyseal marrow, richer in progenitor cell

population [17]. As pointed out by Hurtig, removal of calcified cartilage is an important step in cartilage repair studies in preclinical and clinical situation. Removal of CC provides increased access to progenitor cells. Several studies have shown presence of CC as a barrier to marrow stimulated repair across various species [199, 369, 370]. However, extreme caution needs to be exercised during CC debridement since insult to subchondral bone increases the likelihood of poor repair, subchondral cysts and poor clinical outcome [371-373]. Drilling accompanied with cooling irrigation prevents heat necrosis, bone compaction and ensures removal of bone debris. Increased access to marrow improves the long term repair outcome [21].

Traditionally, the models used in the lab are generated from normal animals where fresh defects are treated to verify the cartilage repair strategies. In reality, the excellent pre-clinical results fail to translate successfully in a clinical setting. The failure may arise due to failed bonding between the repair tissue and surrounding cartilage, impartial restoration of repair tissue or inferior quality of repair tissue. This leads to instability of the newly generated repair tissue finally causing the procedure to fail. Chronic model provides an opportunity to study the impact of the altered joint mechanics on cartilage regeneration. Based on recent studies, it is clear that influence of synovitis, meniscal damage, ligament instability, and altered metabolism in joints cannot be ruled out while studying the pathogenesis of osteoarthritis or evaluation of cartilage repair strategies [374]. Using goat model, Verbout *et al* showed that early intervention proved to be more successful in maintaining normal joint homeostasis (Fig. 2.14). Metabolic alterations including degeneration of matrix and release of matrix components was more pronounced in the defects receiving late treatment. Chronic defects are more challenging to treat as seen by the comparable degeneration observed between untreated and chronic defects. The authors were able to conclude that cartilage regeneration was negatively influenced by the altered joint homeostasis in chronic defects. This study highlights the need for development of chronic defect model for evaluation of cartilage repair strategies in pre-clinical studies. Subchondral bone in chronic cartilage lesions is likely to be denser and more sclerotic accompanied by a reduction in the number as well as chondrogenic potential of progenitor cells [375]. Finally, it also important to evaluate the influence of chronicity of defects on the chondrogenic and osteogenic potential of mesenchymal progenitor cells in the subchondral bone [220]. In conclusion, the evaluation of cartilage repair techniques in chronic defect models is likely to simulate the clinical situation more closely and accurately thereby ensuring the successful translation of these techniques.

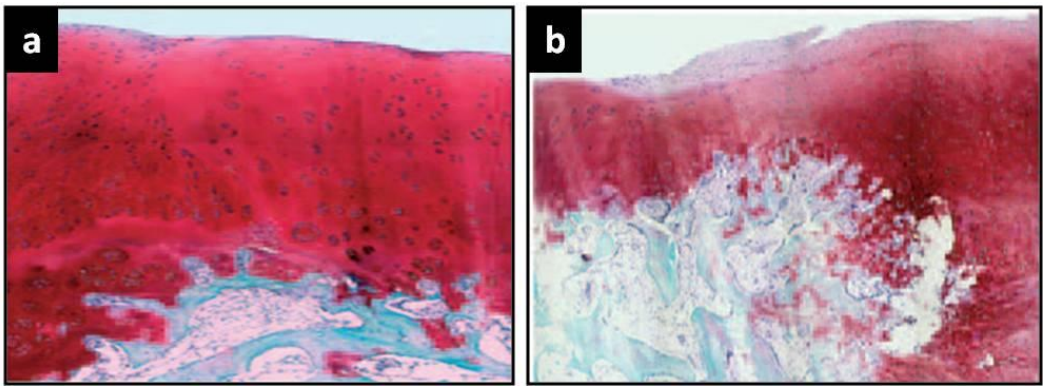

Figure 2.14: Comparison of cartilage repair outcome in groups treated “early” and “late”. (a). Repair response was better in acute defect group characterized by hyaline staining of matrix and evidence of SC bone remodeling. (b). Chronic defects were characterized by incomplete fill, hypertrophy, uneven staining and poor bonding with adjacent cartilage [*Image copied from Saris et al*-374].

Another important aspect while development of animal models is the duration of repair. Early time points are more suitable for monitoring of initial alterations occurring in the defect milieu. The early time point also assists in better understanding of the underlying mechanism of key events participating in the cartilage repair such as cell recruitment, vascularisation and subchondral events [19] with an important bearing on the eventual repair outcome. Moreover, it is important to study the short term outcomes before proceeding to larger studies since poor short term outcomes are rarely translated into successful outcomes in long term [374].

CHAPTER 3 ORGANIZATION OF ARTICLES

The scientific contribution made through this thesis is presented here by means of this section. In previous studies by our group we had found substantial evidence of effect of location of defect on marrow stimulated cartilage repair outcome. In BMS, progenitor cells (Mesenchymal stem cells, MSCs) present in the subchondral bone are recruited into the drill holes and defect to give rise to a repair tissue via a complex cascade of events. The underlying mechanism of high inter-individual variability observed with BMS procedure remains unclear and warrants a better understanding of behavior of individual cells present in subchondral bone. The first part of the study will primarily focus on the characterization of mesenchymal stem cells isolated from condyle and trochlea of young and old rabbit. Since bone marrow stimulation procedure involves cells originating from marrow spaces, bone lining regions and bone; cells will be isolated using collagenase digestion to obtain first population of cells followed by explant culture to obtain second population of cells. The paper is entitled "Bone marrow progenitor cells isolated from young rabbit trochlea are more numerous and exhibit greater clonogenic, chondrogenic and osteogenic potential than cells isolated from condyles". This paper will verify if MSCs exhibit location-dependent variability in their intrinsic biological properties. In addition, the influence of age on the aforementioned biological properties will also be determined. Comprehensive analysis of biological properties will be carried out *in vitro* to compare the cell yield, clonal potential, surface marker profile and potential to differentiate into chondrogenic and osteogenic lineage. This is the first study of its kind aimed at providing valuable insights into our understanding of location and age dependent factors influencing cartilage repair outcome. The paper has been published in the journal "Cartilage".

The results from this study then steered us towards second article where correlation between inherent biological properties of BMSCs and cartilage repair outcome in adult rabbit will be investigated. The results are presented in the article titled "Quality of cartilage repair from marrow stimulation correlates with number clonogenic, chondrogenic and matrix production potential of underlying bone marrow stem cells". This novel study will attempt at exploring the underlying mechanism of donor- and location-dependent variability observed in marrow stimulated repair outcome. Acute defect in skeletally mature rabbits will be created in condyles and trochlea in one knee and repaired by BMS by microdrilling. The contralateral knee will be used for isolation of cells from condyle and trochlea followed by *in vitro* characterization of their biological properties. Characterization of repair tissue and BMSCs will help us establish influence of location and donor

on the cartilage repair outcome. In the most important step, the study will seek after donor- and location-dependent correlation between cartilage repair outcome and inherent properties of stem cells present in condyle and trochlea using suitable statistical models. This paper has been submitted to the journal “Osteoarthritis and Cartilage”.

These two studies helped in establishing the donor-, age- and location-dependent factors with a significant impact on the outcome and variability of cartilage repair outcome. Inspired from our analysis of underlying mechanism of variation in cartilage repair, we finally proceeded to the final stage of this study, the results from which have been presented in the article titled “Freeze-dried chitosan/PRP implants improve marrow stimulated cartilage repair in a chronic defect rabbit model”. Clinical translation of several cartilage repair strategies with promising preclinical results is unsuccessful since cartilage lesions in clinical situation are rarely detected in early stages and have already progressed to degenerative, chronic stage by the time surgical intervention is carried out. With this issue in mind, this paper will explore critical aspects in cartilage repair using a challenging chronic defect model to analyse the influence of augmented marrow stimulation on repair response. Chronic trochlear defects will be developed in skeletally mature rabbits and treated by microdrilling for BMS. The impact of stimulus provided by means of chitosan and PRP on BMSCs contributing to marrow stimulated cartilage repair will be examined. This article has been submitted to the journal “Biomaterials”. The studies presented in these three articles not only helped us in recognizing critical factors implicated in variability in marrow stimulated repair outcome but also present a promising approach to address the critical clinical osteoarthritic situation.

CHAPTER 4 ARTICLE 1: BONE MARROW PROGENITOR CELLS ISOLATED FROM YOUNG RABBIT TROCHLEA ARE MORE NUMEROUS AND EXHIBIT GREATER CLONOGENIC, CHONDROGENIC AND OSTEOGENIC POTENTIAL THAN CELLS ISOLATED FROM CONDYLES

Bone marrow stimulation initiates repair by fracturing or drilling into subchondral bone at the base of a debrided cartilage defect, typically leading to the formation of a fibrocartilagenous repair tissue. The common drawbacks of the procedure include incomplete regeneration, high inter-individual variability and poorer outcome in older animals. Based on evidence in literature, we believe that cartilage repair outcomes are affected by defect location and age of donor, suggesting that the biological characteristics of progenitor cells (Bone Marrow Progenitor cells, BMPCs or mesenchymal stem cells; MSCs) present in underlying subchondral bone strongly influence the repair response. Chapter 4 focuses on comprehensive analysis of the BMPCs/MSCs present in rabbit condyle and trochlea, taking into account location and age. In this study, we established isolation and culture protocols for BMPCs/MSCs derived from rabbit distal femur. BMPCs/MSCs were isolated from condyles and trochlea of young and old rabbits using cultures derived from collagenase-digests of marrow stroma and explant culture of digested bone. Characterization of BMPCs/MSCs was primarily based on ISCT guidelines with assays added for better understanding of their inherent properties. P0 cell yield was used an indicator of initial population of cells present in subchondral bone. CFU-f assay provided an estimation of their clonogenic potential and thus stemness. Surface marker expression profile was carried out to confirm the composition of population as well as verify influence of age and location. Finally, cells were differentiated into osteogenic and chondrogenic phenotype. *In vitro* analysis of properties of BMPCs/MSCs is expected to shed light on the differences in inherent properties of BMPCs/MSCs present in condyle and trochlea including cell population, clonogenic potential and differentiation potency. We believe that the extensive characterization of BMPCs/MSCs presented here will improve our understanding of mechanism underlying the location-dependent variability in marrow stimulated repair outcome. By understanding the impact of ageing on the properties of BMPCs/MSCs, our results will also help in adapting the procedure to better suit the needs of older population. In Press, *Cartilage.*

Bone marrow progenitor cells isolated from young rabbit trochlea are more numerous and exhibit greater clonogenic, chondrogenic and osteogenic potential than cells isolated from condyles

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Abstract

Objective: Bone marrow stimulation procedures initiate repair by fracturing or drilling subchondral bone at base of cartilaginous defect. Earlier studies have shown that defect location and animal age affect cartilage repair outcome, suggesting a strong influence of structural and biological characteristics of subchondral bone. Here we analyzed comprehensive biological characteristics of bone marrow progenitor cells (BMPCs) in subchondral bone of young and old rabbit condyle and trochlea. We tested the hypothesis that *in vitro* biological properties of BMPCs are influenced by location, age of donor and method of their isolation.

Design: In vitro biological properties including cell yield, colony-forming unit fibroblasts (CFU-f), surface marker expression and differentiation potential were determined. Comparisons were carried out between trochlea versus (vs.) condyle and epiphyseal versus metaphyseal bone using old (N=5) and young animal knees (N=8) to generate collagenase and explant-derived BMPC cultures.

Results: CFU-f, cell yield, expression of stem cell markers and osteogenic differentiation were significantly superior for younger animals. Trochlear subchondral bone yielded the most progenitors with the highest clonogenic potential and cartilaginous matrix expression. Trochlear collagenase-derived BMPCs had higher clonogenic capacity than explant-derived. Epiphyseal cells generated a larger chondrogenic pellet mass than metaphyseal-derived BMPCs. All older pellet cultures and 1 non-responder young rabbit failed to accumulate glycosaminoglycans (GAGs).

Conclusion: Taken together, these results suggest that properties intrinsic to subchondral progenitors could significantly influence cartilage repair potential, and could partly explain variety in cartilage repair outcomes using same cartilage repair approach.

4.1 Introduction

Articular cartilage present at the ends of long bones possesses very limited regenerative capacity and is frequently repaired using bone marrow stimulation typically leading to formation of fibrocartilaginous repair tissue. Channels created in subchondral bone, provide access to underlying bone marrow stroma rich in BMPCs which are recruited to cartilage defect site. They proliferate and differentiate into a chondrogenic phenotype leading to formation of a repair tissue with varying amounts of hyaline and fibrous cartilage [1]. In addition to mechanical and structural factors; differences in number, stemness and differentiation potential of BMPCs are expected to influence cartilage repair. These properties can be characterized *in vitro* using assays for determination of cell yield, colony forming unit-fibroblast potential (CFU-f), expression of cell surface markers and multilineage differentiation potential.

Previous studies have reported an influence of the defect location and age on cartilage repair outcome, suggesting a strong influence of biological characteristics of subchondral BMPCs [2-4]. 34-58% of chondral injuries are observed in condyles while trochlea accounts for 6-8% cases [2]. Whereas human trochlear defects are harder to treat compared to condylar defects-likely due to anatomical and mechanical dissimilarities- [3-5], animal models present varying scenarios. While trochlea demonstrated superior repair potential in rabbit [3, 4]; quality of repair in a sheep model [2] was better in condyle as shown in earlier studies by our group reflecting important species- and site- dependencies. Primary goal of this study was to verify influence of location on *in vitro* properties of rabbit BMPCs which might impact *in vivo* repair outcome. Earlier, our group also showed that drilling to 6mm vs 2mm improves repair outcome in trochlea [6], possibly since it provides access to the metaphyseal red marrow rich in BMPCs. Therefore, we compared *in vitro* biological properties of BMPCs in upper and lower regions of trochlea in order to evaluate the influence of the depth of drilling on the repair response in clinical situations. We also explored influence of age on BMPC properties to improve our understanding of underlying causes of poor repair in older individuals. Finally, BMPCs were isolated from stromal spaces and bone lining regions (collagenase digestion) as well as outgrowths from explants since cells participating in bone marrow stimulated cartilage repair originate from both these sites. Human trabecular cells reacted positive for Stro-1 and CD105 and negative for CD34 and CD45 suggesting presence of multipotential cells in bone [7]. Cells remaining in explants

most likely migrate out after collagenase treatment and demonstrate potential to form colonies [8-10] and form new bone and cartilage *in vivo* [11]. We optimized the concentration and duration of enzyme treatment for maximum yield and viability since collagenase digestion is a harsh treatment compared to explant culture with a damaging effect on *in vitro* properties of BMPCs [12, 13]. Owing to similar cell surface marker profile and trilineage differentiation potential, we expect that collagenase- and explant derived BMPCs would demonstrate comparable *in vitro* biological properties.

Through this study, a comprehensive analysis of biological characteristics of BMPCs from young and old rabbit condyle and trochlea was performed to verify influence of location, age and isolation method on their *in vitro* biological properties. The specific hypotheses tested were: 1) Cell yield, stemness markers, clonogenic, chondrogenic and osteogenic potential are superior in trochlea compared to condyles. 2) Cell yield, stemness markers, clonogenic, chondrogenic and osteogenic potential decrease with age. 3) There is no difference in *in vitro* biological properties of collagenase and explant-derived BMPSCs.

4.2 Materials and Methods

4.2.1 Necropsy

Animal study protocols were approved by University of Montreal institutional committee "Comité de déontologie de l'expérimentation sur les animaux". Female New Zealand White rabbits -young (4 months; n=8 knees) and retired breeders (12-36 months; n=5 knees) were used. Rabbits were anaesthetized with xylazine-ketamine and euthanized by an overdose of sodium pentobarbital. Both femurs were exposed, dissected of all soft tissues, and placed in ice-cold DMEM/F12 medium.

4.2.2 Isolation of BMPCs

Reagents were obtained from Sigma-Adrich (Oakville, ON, Canada) unless otherwise specified. Under sterile conditions, bone was rinsed with HBSS-Penicillin-Streptomycin (P-0781). Condylar and trochlear bone segments were obtained using isomet saw (Fig. 4.1a-1d). Trochlear segments were separated into Trochlea Upper (epiphyseal bone) and Trochlea Lower (metaphyseal bone) separated by growth plate in case of young animals or epiphyseal line in retired breeders (Fig. 4.1e). Following further fragmentation into 4-5 mm chips using flat blades (Fig. 4.1f), chips were weighed in order to determine cell yield/wet mass at end of P0. Bone chips were treated with 3 mg/ml collagenase type-XI (C-9697) for 2 hours/37^{o}C/100rpm. Post-digestion cell suspension was centrifuged and cells were used to obtain first population of BMPCs-"collagenase-derived". Digested bone chips were rinsed twice with HBSS to remove residual collagenase and placed in flasks for 4 days to generate second population of cells-"explant-derived". Colonies of fibroblast-like-cells were cultured in growth medium comprised of DMEM/F12 (Gibco, 12500-062) containing 10% FBS and 1X penicillin-streptomycin solution. Following first medium change at 4-5 days to remove dead and non-adherent cells, medium was changed every 2-3 days.

4.2.3 Histology of bone chips

Two bone chips were collected at three stages-before enzyme treatment (D0, pre-digested), immediately after enzyme treatment (D0, post-digested) and day 4 of explant culture (D4, post-digested) for evaluation of collagenase digestion efficiency and migration of cells from explants. They were fixed in 10% normal buffered formalin (Fisher), decalcified in 0.1N HCl/0.1% glutaraldehyde, embedded in paraffin, sectioned at 5 μm and stained with Hematoxylin (Fisher, SH30-500D) and Eosin (Surgipath, CI 45380).

4.2.4 Cell Yield

For accurate comparison of initial BMPC population isolated from different locations of bone, all P0 samples were trypsinized at the same time - when at least one of the samples reached 80-85% confluency - irrespective of other cultures' confluency. While sufficient cells were obtained from all 3 locations in all 8 young knees, only 3 of older knees generated substantial cell yield for statistical analysis (n=8 knees and n=3 knees for young and old animals respectively). The cultures were trypsinized at d6-7 (collagenase-derived) and d10-11 (explant-derived) in young and d8-10 (collagenase-derived) and d11-13 (explant-derived) in older animals. Cultures were passaged twice using 0.25% Trypsin-EDTA (Invitrogen, 25200-072). Cell viability was determined with Trypan Blue exclusion and Countess system (Invitrogen). Cell yield was determined as follows: P0 cell yield (10^6/g) = viable cell count (10^6)/ wet weight of bone chips (g)

4.2.5 Flow cytometry

P1 BMPCs (10^6/ml) were blocked with 1% solution of BSA-PBS (A-7888) followed by incubation with non-conjugated monoclonal antibodies and goat anti-mouse IgG-FITC. MPC positive antigens used were CD29 (Millipore, MAB1951Z) and CD44 (Cell Marque, 144M-96). Hematopoetic marker CD34 (Cell Marque, 134M-16) and pan-leukocytic marker CD45 (Cell Marque, 145M-97) were used as negative markers. Mouse IgG isotype primary antibody served

as negative control. Stained cells were acquired using MoFlo cytometer and data was analysed using Summit software after appropriate gating to exclude cell debris.

4.2.6 CFU-f

P1 cells were trypsinized, replated as single cells at low density of 100 cells/well and cultured in complete growth medium for 6-7 days. Cultures were stained with 1% (w/v) solution of crystal violet (C-0775) and colonies containing more than 50 cells were counted.

4.2.7 Chondrogenic differentiation assay

Chondrogenesis was induced in 3-dimensional high density pellet cultures of BMPCs using P2 cells. 0.25×10^6 cells (in DMEM/F-12) were centrifuged in 15 ml polypropylene tubes at 300g/5 minutes to form pellets. Tubes were placed at 37°C/5% CO_2 with caps loosened to allow gaseous exchange. Next day, pellets were switched to 0.5 ml chondrogenic medium comprised of serum-free high-glucose DMEM (Gibco, 12100-046) supplemented with dexamethasone (100nM, Cat. No. D-2915), ascorbate (50 µg/ml, A-8960), ITS (1X), sodium pyruvate (100ug/ml, Gibco) and BSA (1.25mg/ml, A-7888) for three weeks. Ascorbate was added fresh at the time of medium change on alternate days. Control and stimulated pellets were cultured in absence and presence of TGF-βIII (R&D, FB2712091) respectively. Low magnification images of fixed pellets were used to determine their gross structure and diameters (used as an indicator of amount of matrix synthesized) using Northern Eclipse software (Empix Imaging).

4.2.8 Histology of pellets

Pellets were fixed in 4% paraformaldehyde/0.1M sodium cacodylate/2.5%w/v cetylpyridinium chloride followed by sucrose infiltration, OCT embedding and sectioned at 8 µm using CryoJane tape-transfer cryosectioning system. Sections were stained with Safranin-O (Fisher, S670-25) and Fast Green (F-7252) to detect GAGs as previously described [14]. Collagen type-II expression was determined by immunostaining. Sections were treated with hyaluronidase (H-

3506) and pronase (P-8811) and blocked with goat serum (G-9023) followed by anti-collagen-type II (clone II6B3, DHSB, 1:10) antibody. Following treatment with biotinylated secondary antibody (B-7151) and Vectastain ABC kit (Vector, Cat. No. AK-5000), alkaline phosphate red substrate kit (Vector, SK-5100) was used to develop the stain. Images of Safranin O/Fast Green-stained sections were scored using a 0 to 4 system by one blinded observer (AC). A chondrogenesis score of 0 was attributed to pellets which had no Safranin-O staining (Panel-a in Fig. 4.6). Chondrogenesis score of 4 was given to pellets which had a matrix that was rich in GAG and had cells with characteristic chondrocyte and hypertrophic chondrocyte morphology (Panel-b of Fig. 4.6).

4.2.9 Osteogenic differentiation assay

P2 cells were trypsinized and replated in 24-well plates and cultured in complete growth medium. Confluent monolayers were stimulated with osteogenic medium comprised of α-MEM (Gibco. 12000-014) supplemented with dexamethasone (100nM, D-2915), ascorbic acid (L-) 2-phosphate sesquimagnesium salt (100µM, A-8960) and β-glycerophosphate (5mM, G-9422). Control cultures were cultured in absence of dexamethasone. Presence of osteogenic matrix was determined after four weeks by Alizarin Red S (AR) (A5533) staining. Absorbance of 10% acetic acid extract was recorded at 425 nm and used to quantify AR present in each sample using AR standard curve.

4.2.10 Statistical Analysis

Statistical analysis was performed using SAS Enterprise Guide 5.1 and SAS 9.3. Since several segments were collected from both legs of each rabbit, mixed model was used to account for influence of donor and age (n=8 young knees and n=3 or 5 old knees). Fixed effects were location (condyles, upper trochlea or lower trochlea), age (young or old), isolation method (collagenase- or explants-derived cultures), and treatment (for chondrogenic or osteogenic stimulation only), while donor was a random effect. Data in figures are presented as mean (circle); median (line); Box: 25^{th} and 75^{th} percentile;

Whiskers: Box to the most extreme point within 1.5 interquartile range.

4.3 Results

4.3.1 BMPCs can be isolated from different locations in rabbit femur

Bone fragments displayed marrow stroma rich in cells before digestion (Fig. 4.1g-i) following which variable fractions of bone marrow stroma were released into supernatant and marrow spaces became clearer (Fig. 4.1j-l). Bone lining cells seen in pre-digested samples (arrows in Fig. 4.1g-i) were no longer observed in post-digestion samples (Fig. 4.1j-l). Numerous lacunae were observed in bone matrix after 4 days of explant culture (dashed arrows in Fig. 4.1j-l). Upon seeding, BMPCs readily separated from cells of hematopoietic origin owing to their adherence to tissue culture plastic.

In both collagenase and explant cultures, cells adhered as rounded cells, acquired characteristic fibroblastic morphology later and grew as colonies in P0 (Fig. 4.1m and n). No apparent morphological difference was observed in cells from condyles, upper trochlea and lower trochlea.

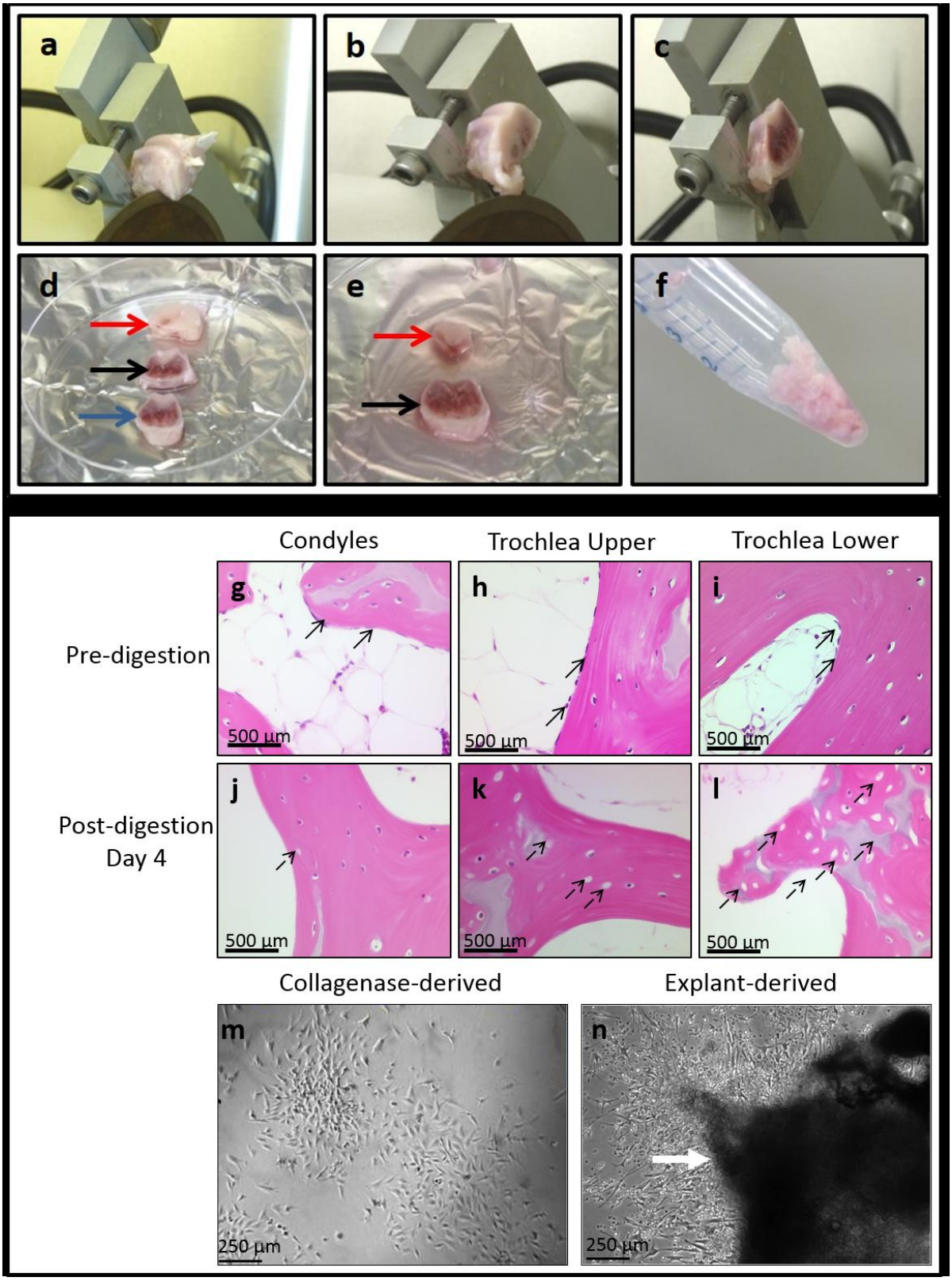

a
b
c
d
e
f
Condyles
Trochlea Upper
Trochlea Lower
Pre-digestion
Post-digestion
Day 4
g
h
i
j
k
l
500 µm
Collagenase-derived
Explant-derived
m
n
250 µm

Figure 4.1: Processing of rabbit femur. Femoral end secured on sample holder of Isomet saw while condylar segment is being sectioned off (a). Femoral end while distal trochlear segment is being sectioned off (b). Femoral end while proximal trochlear segment is being sectioned off (c). Three segments post-sectioning: Condyles, distal trochlea and proximal trochlea (d). Separation of a trochlear segment into Upper and Lower trochlea above and below the growth plate (in the young) and epiphyseal line (in the old) (e) All segments were fragmented with flat blades to yield 3 groups: Condyles, Upper trochlea (pooled from distal and proximal segments) and Lower trochlea (pooled from distal and proximal segments) (f). H&E stained paraffin sections of bone fragments were collected at pre-digestion (g-i) and post-digestion after 4 days of explant culture (j-l). Marrow spaces are initially occupied by cells. Arrows point to the bone lining cells in pre-digested samples (g-i). In post-digestion samples, marrow spaces are partly void, bone-lining cells are mostly absent. Dashed arrows point to empty bone lacunae in post-digestion samples (j-l). Bone marrow progenitor cells growing as colonies from Collagenase-derived cultures (m) and Explant-derived cultures (n). Arrow in n points to an explant bone fragment.

4.3.2 Cell yield, clonogenic potential and matrix production are highest in trochlear segments; and decrease with age

In young animals, trochlea upper showed higher cell yield versus condyles for both collagenase ($p<0.0001$) and explant-derived cultures ($p<0.0001$) (Fig. 4.2a). In older animals, cell yield was highest for trochlear upper and lowest for condyles in collagenase-derived ($p=0.0041$) (Fig. 4.2b). Cell yield was lower in old versus young animals, significantly for explant-derived cultures ($p = 0.028$) (Fig. 4.2a, b). Yield for collagenase- and explant-derived BMPCs was found to be comparable in young animals (Fig. 4.2a), while poorer yield was observed for explant-derived cultures in old animals ($p = 0.0022$) (Fig. 4.2b).

All samples expressed BMPC markers CD29 and CD44 (Fig. 4.2c, d) with no apparent difference between condyles, trochlea upper and trochlea lower or collagenase- and explant-derived BMPCs. Substantial difference was observed between young and old animals (Fig. 4.2c vs. 4.2d). Expression of BMPC markers was poor in older animals with a considerable proportion of cells

showing no expression. All samples lacked expression of CD34 (Fig. 4.2e, f) and CD45 indicating absence of cells of hematopoetic or leukocytic origin.

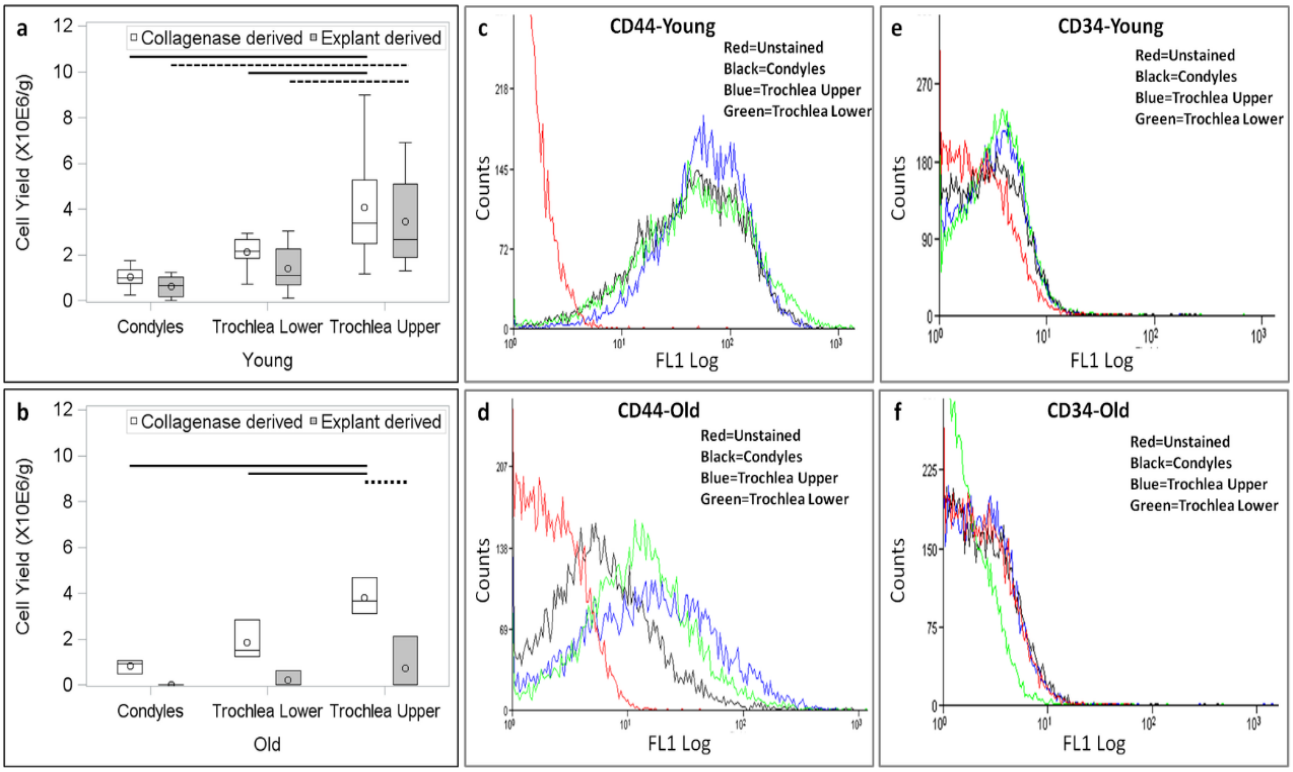


Figure 4.2: Cell yield observed in young animals (a, n = 8 knees) versus old animals (b, n = 3 knees) for collagenase- and explant-derived cultures. Cell yield was higher for Trochlea Upper in both collagenase- and explant-derived cultures in the young animals (a). Cell yield was also higher for Trochlea Upper in collagenase-derived samples in older animals (b). Data is presented as mean (circle); median (line); Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. Horizontal lines show significant differences between pairs. Greater expression of stem cell marker CD44 was observed in young animals (c) versus old animals (d). CD34 was not expressed in young (e) or old (f) animals.

CFU-f was strongly influenced by location, age and method of isolation (Fig. 4.3a-l and m, n). Upon macroscopic examination, trochlear colonies appeared more numerous and larger (Fig. 4.3b, c vs. 4.3a and 4.3e, f vs. d). In young animals, both collagenase-derived trochlear segments had higher CFU-f than condylar segments indicating greater clonogenic potential (Fig. 4.3m)

(p=0.02-trochlea upper and p<0.0001-trochlea lower respectively). CFU-f was significantly lower in older animals for both collagenase (p=0.02) and explant-derived BMPCs (p=0.06). Finally, explant-derived BMPCs showed inferior clonogenic potential versus collagenase-derived BMPCs in young and old animals indicated by lower CFU-f (Fig. 4.3m, n) (p<0.0001).

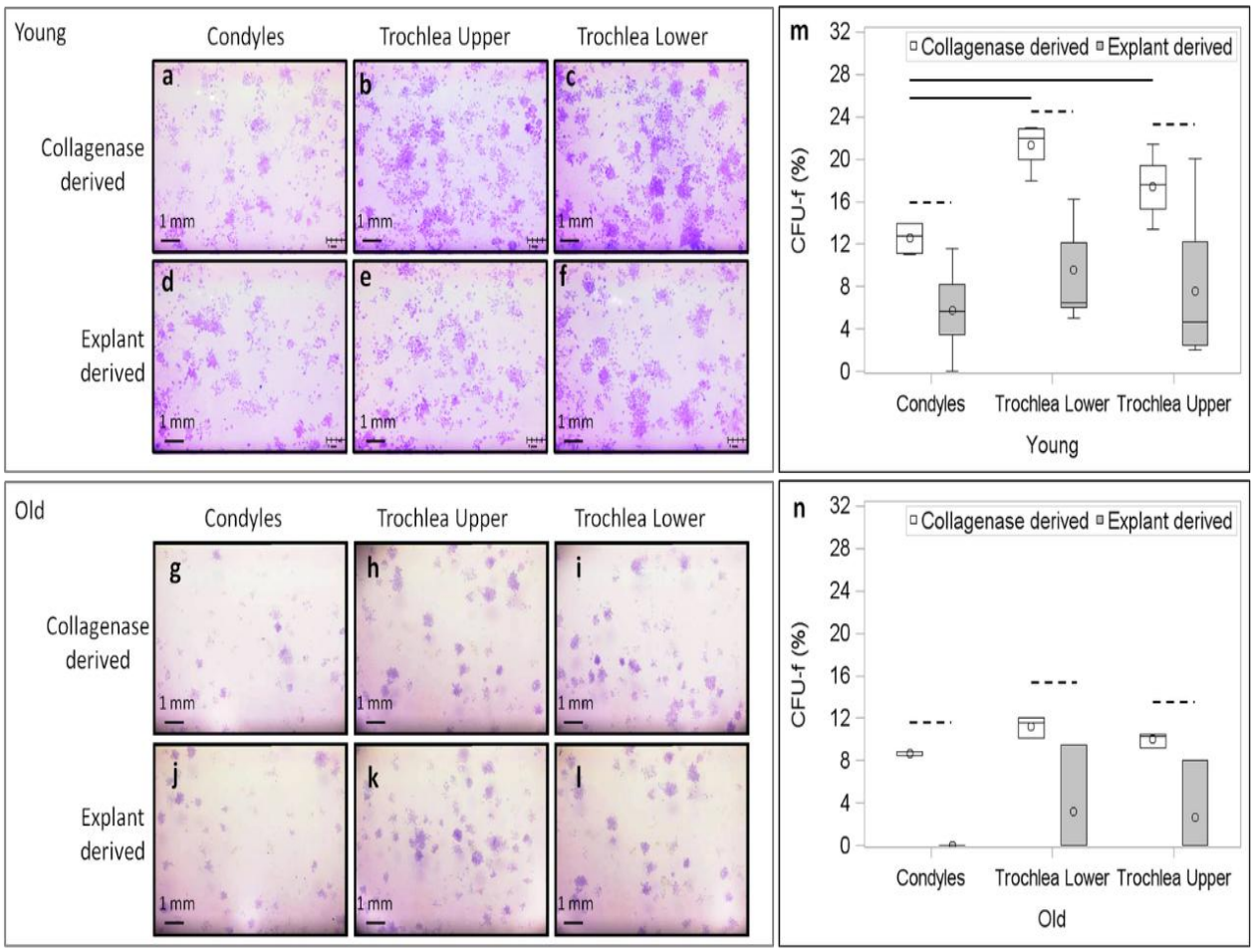
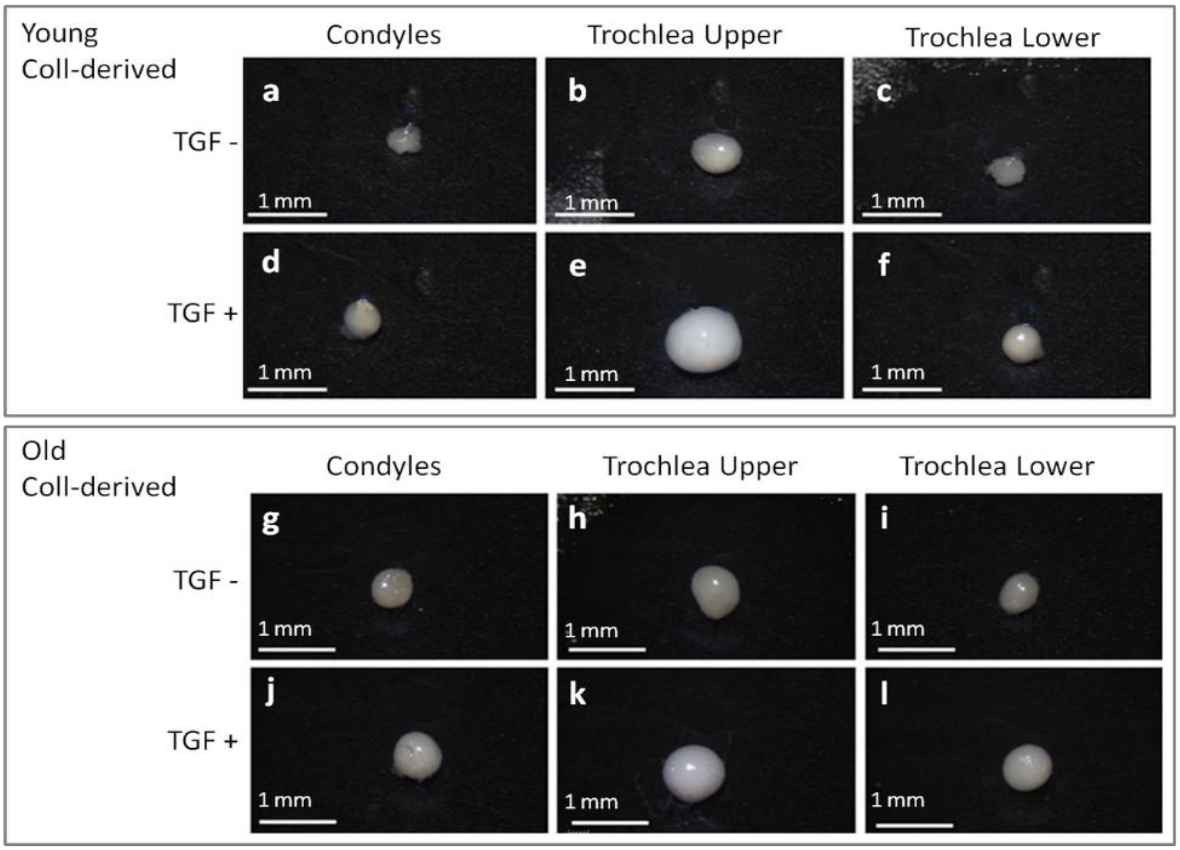


Figure 4.3: Clonogenic colonies stained with 1% (w/v) Crystal Violet stain show increased colony formation in the case of young (panels a to f) versus old animals (panels g to l). Higher CFU-f was observed for both trochlear segments versus condyles in collagenase-derived cultures in young animals (m, n = 8 knees), but not in old animals (n, n = 3 knees). CFU-f was consistently higher for collagenase-derived cultures compared to explant-derived cultures (m & n). Data is presented as mean (circle); median (line); Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. Horizontal lines show significant differences between pairs.

In young animals, pellets cultured without TGFβ-III were smaller than those cultured in presence of TGFβ-III in both collagenase (Fig. 4.4a-c vs. d-f) and explant-derived cultures ($p<0.0001$) (Fig. 4.4 g-l). Pellets from upper trochlear BMPCs were largest in both collagenase ($p=0.1$) and explant-derived cultures ($p=0.03$) indicating maximum matrix production (Fig. 4.4m, n). Respective pellets from collagenase- and explant-derived BMPCs from same location were similar in size (Fig. 4m vs. n) (data not shown for explant-derived cultures).


Young
Coll-derived
Condyles
Trochlea Upper
Trochlea Lower
TGF -
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a
b
c
d
e
f
1 mm
Old
Coll-derived
g
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i
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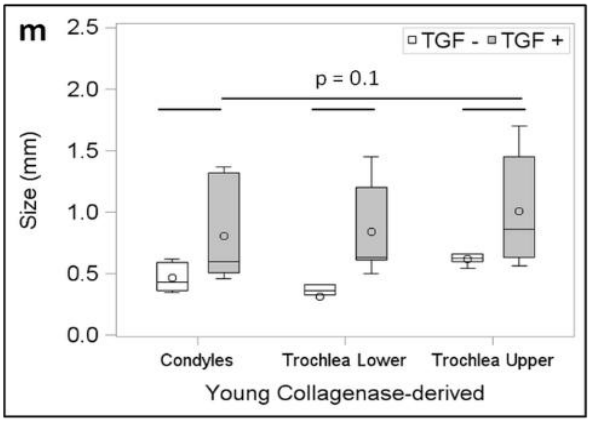
l


m
TGF - TGF +
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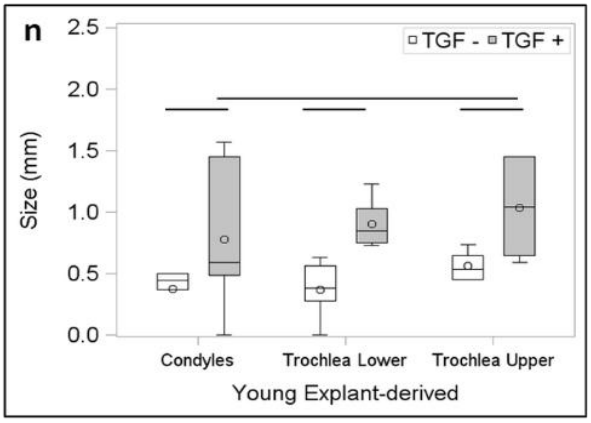
Condyles
Trochlea Lower
Trochlea Upper
Young Collagenase-derived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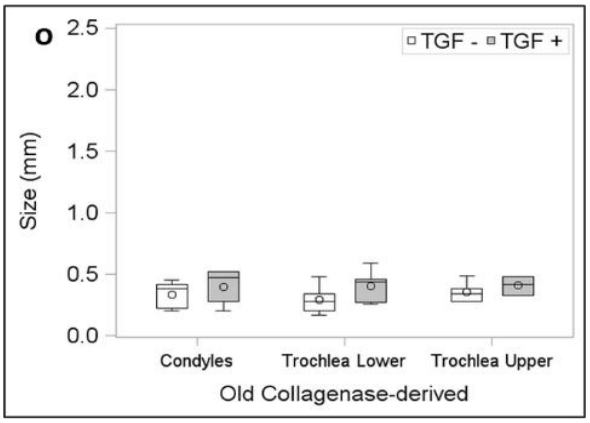
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TGF - TGF +
Size (mm)
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Trochlea Upper
Old Collagenase-derived

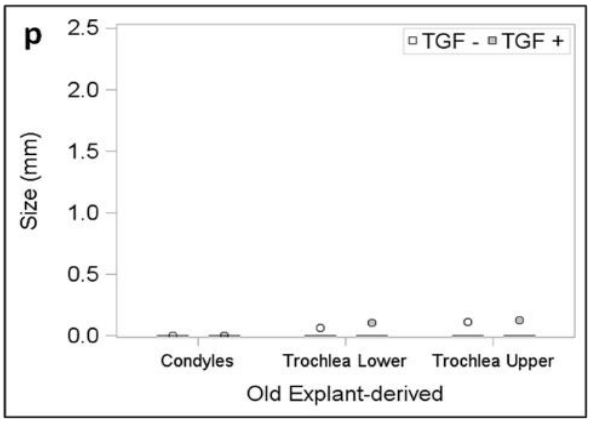

p
TGF - TGF +
Size (mm)
Condyles
Trochlea Lower
Trochlea Upper
Old Explant-derived

Figure 4.4: Gross structure of pellets show increase in size upon culture in presence versus absence of TGF-βIII in young animals (a-f) but not in old animals (g to l). Pellets derived from collagenase-derived cultures are shown here. Pellets from trochlea upper samples were larger compared to pellets from condyles in young animals (m&n, n = 6 knees), and significantly so in the case of explant-derived cultures (n). All samples show low pellet diameter in old animals for collagenase- (o, n = 5 knees), and even lower in explant-derived cultures (p, n=2 knees). Data is presented as mean (circle); median (line); Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. Horizontal lines show significant differences between pairs, unless otherwise indicated.

4.3.3 Chondrogenic potential displays high inter-individual variability, is on average superior for trochlear segments and decreases with age

Pellets cultured without TGF-βIII lacked expression of GAGs (Fig. 4.5) as well as Coll-II (results not shown). In young animals, enormous variation was observed in quality of matrix produced by pellets cultured in presence of TGF-βIII. In one young animal-a high responder- pellets expressed high levels of GAGs (Fig. 4.5-panels a&b) and Coll-II. Two young animals were labeled medium-responders (Fig. 4.5-panels c&d). In another young animal-a low responder- very little chondrogenesis was observed (Fig. 4.5-panels e&f). While chondrogenesis scores reflect this variation and have large spread, on average, both collagenase- and explant-derived BMPCs derived from trochlea upper displayed increased chondrogenic potential (Fig. 4.5-i&j). By contrast, outcome in older animals was more consistent (Fig. 4.5-panels g&h). Poor matrix with negligible expression of GAG (Fig. 4.5-panels g&h) and Coll-II and corresponding low chondrogenesis scores (Fig. 4.5-k&l) was observed for all samples cultured in presence of TGF-βIII, irrespective of location or method of isolation.

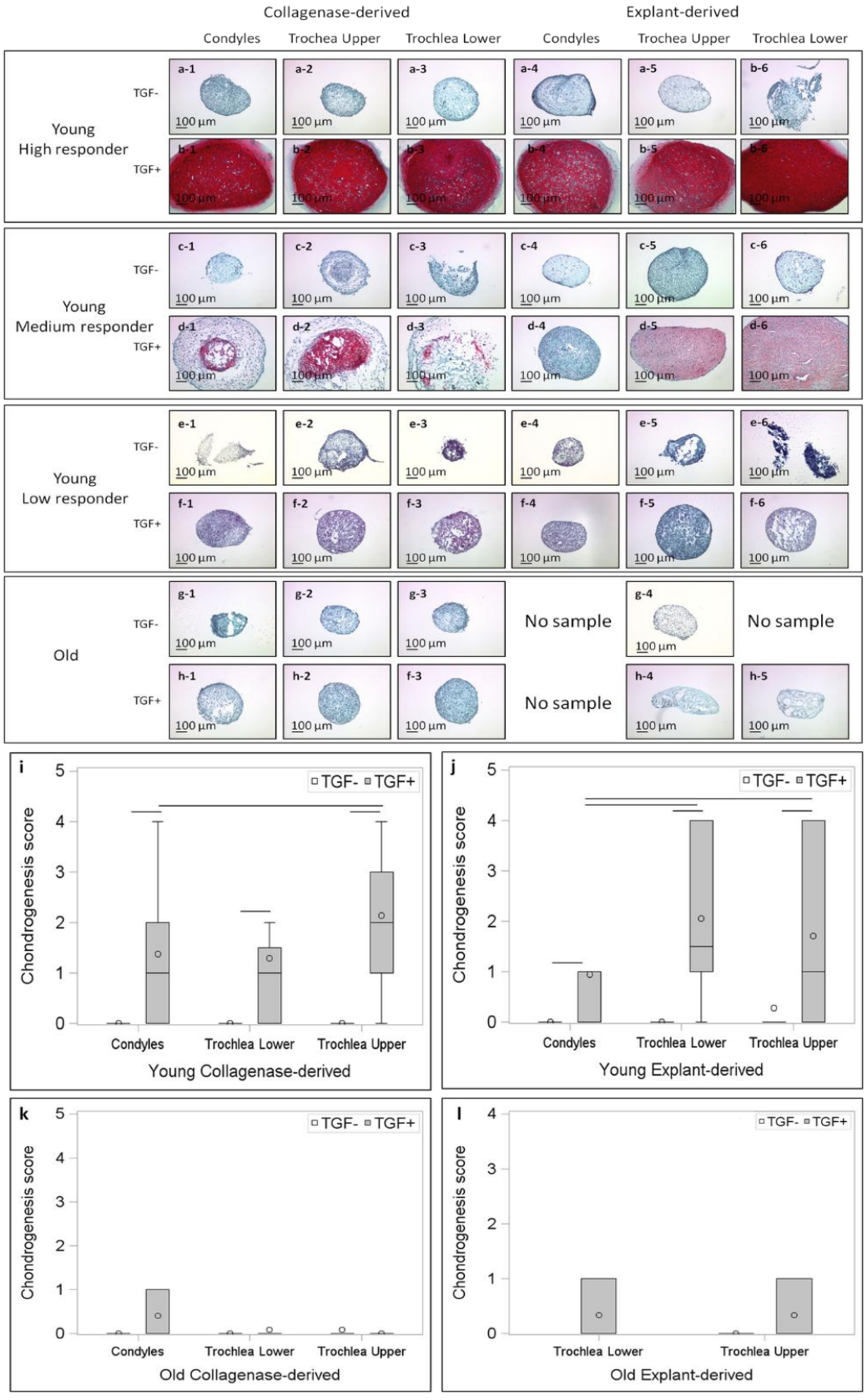
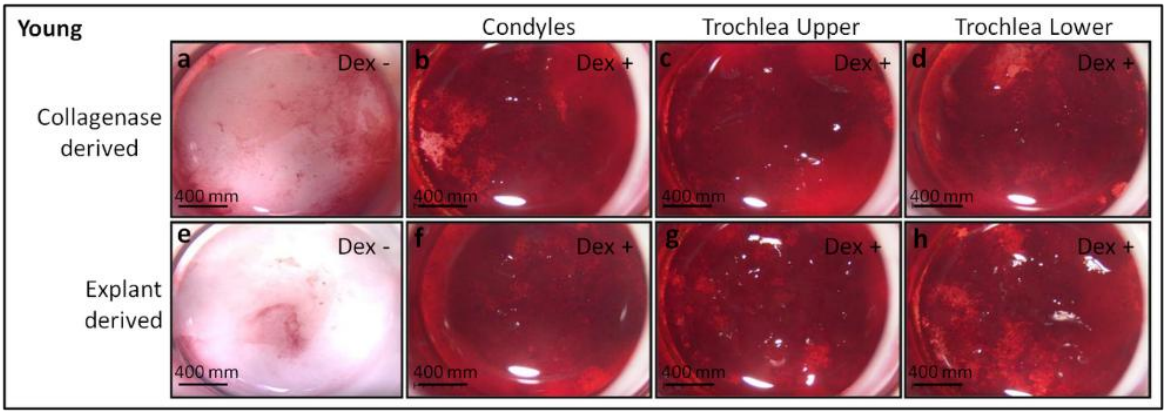
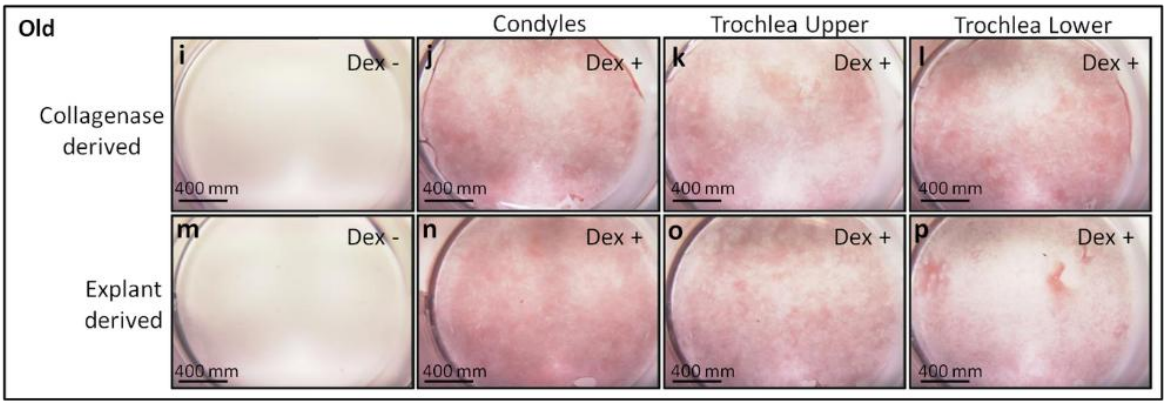

Collagenase-derived
Explant-derived
Condyles
Trochea Upper
Trochlea Lower
Condyles
Trochea Upper
Trochlea Lower
Young
High responder
Young
Medium responder
Young
Low responder
Old
TGF-
TGF+
100 µm
No sample
No sample
No sample
i
j
k
l
Chondrogenesis score
TGF- TGF+
Condyles
Trochlea Lower
Trochlea Upper
Young Collagenase-derived
Young Explant-derived
Old Collagenase-derived
Old Explant-derived

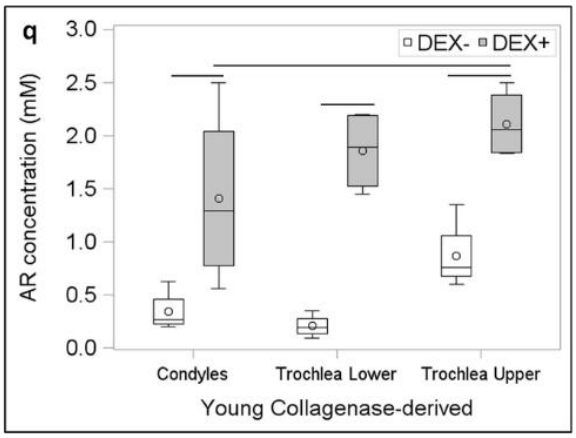
Figure 4.5: Safranin O/Fast Green staining of pellets derived from collagenase- and explant-derived cultures, showing a high responder (panels a&b), medium responder (panels c&d) and low responder (panels e&f) in the young, as well as pellets derived from an older animal (panels g&h). TGF-βIII treatment only induced chondrogenesis in the young animals, as revealed by the chondrogenesis score (compare panels i&j to panels k&l). Cells derived from the trochlear upper sample displayed increased chondrogenic potential in both collagenase-derived and explant-derived cultures in the young (i&j). Data is presented as mean (circle); median (line); Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. Horizontal lines show significant differences between pairs.

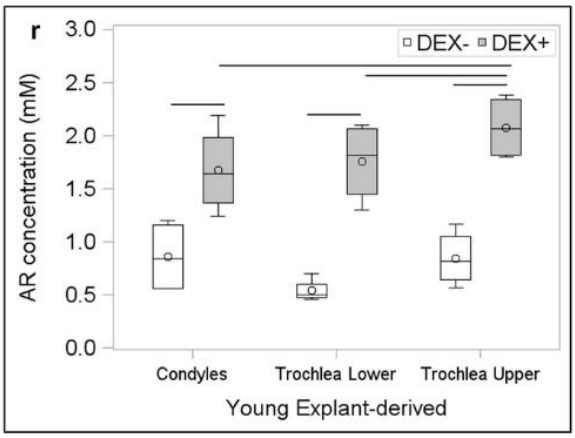
4.3.4 Osteogenic potential is higher in trochlear segments and decreases with age

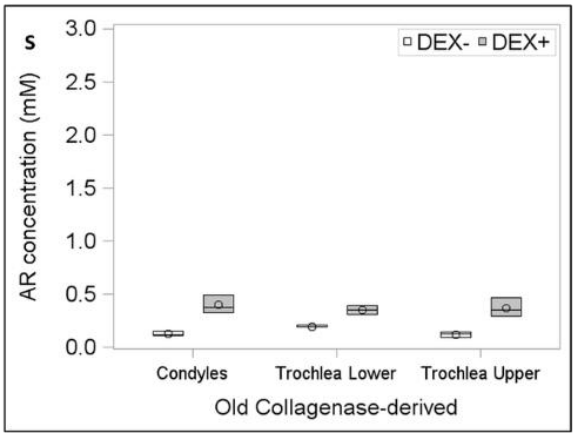
Osteogenic matrix expression was significantly influenced by age, location and treatment with dexamethasone. In young animals, cultures differentiated into osteogenic phenotype upon dexamethasone stimulation and matrix calcification was much stronger in presence of dexamethasone ($p<0.0001$) (Fig. 4.6 a, e vs 4.6b-d and 4.6f-h). Staining observed with AR was superior in trochlea upper for collagenase- ($p<0.0001$) and explant-derived cultures ($p=0.0154$) (Fig. 4.6q, r), while no apparent difference was observed between collagenase- and explant-derived samples (Fig. 4.6q, r). Osteogenic differentiation was found to be attenuated with increasing age and even when cultured in presence of dexamethasone, a very poor osteogenic matrix was observed in case of old animals (Fig. 4.6j-l, 4.6n-p). AR extracted from cultures from older animals was much lower in all cases signifying poor osteogenic differentiation and no significant influence of location (Fig. 4.6s, t) or method of isolation (Fig. 4.6s vs 4.6t) was observed.


Young
Condyles
Trochlea Upper
Trochlea Lower
Collagenase derived
Explant derived
a Dex -
b Dex +
c Dex +
d Dex +
e Dex -
f Dex +
g Dex +
h Dex +
400 mm


Old
Condyles
Trochlea Upper
Trochlea Lower
Collagenase derived
Explant derived
i Dex -
j Dex +
k Dex +
l Dex +
m Dex -
n Dex +
o Dex +
p Dex +
400 mm


q
DEX- DEX+
AR concentration (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Condyles
Trochlea Lower
Trochlea Upper
Young Collagenase-derived


r
DEX- DEX+
AR concentration (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Condyles
Trochlea Lower
Trochlea Upper
Young Explant-derived


s
DEX- DEX+
AR concentration (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Condyles
Trochlea Lower
Trochlea Upper
Old Collagenase-derived

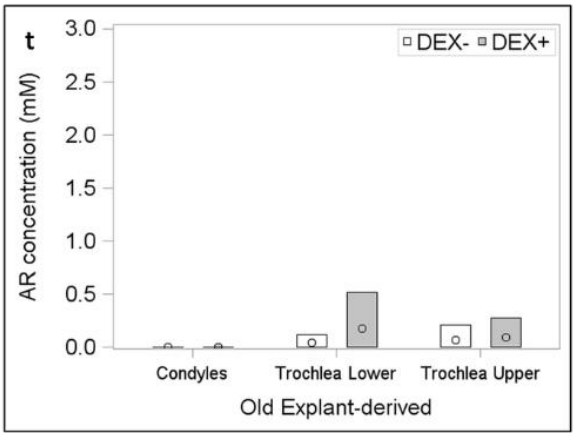

t
DEX- DEX+
AR concentration (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Condyles
Trochlea Lower
Trochlea Upper
Old Explant-derived

Figure 4.6: Osteogenic cultures stained with Alizarin Red S (AR) showed increased matrix synthesis in presence (panels b-d&f-h) versus absence (a&e) of dexamethasone in young animals. Poorer osteogenic matrix synthesis was found in old animals (i-p). In young animals (n = 4 knees), AR accumulation was higher in upper trochlea samples in both collagenase-(q) and explant-derived cultures (r). Inferior AR accumulation was found in old animals (s&t, n=3 knees). Data is presented as mean (circle); median (line); Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. Horizontal lines show significant differences between pairs.

Table 4.1: Influence of location, age and isolation method on the different *in vitro* biological properties assessed.

Property assessed	**Influence of location**	**Influence of age**	**Influence of isolation method**
Cell yield	Higher in upper trochlea in young animals	Decreases with age	Lower in explant-derived in old animals
Significant difference (Y/N)	Y for collagenase- and explant-derived cultures in young	Y for collagenase- and explant-derived	Y
Expression of markers	No effect	Decreases with age	No effect
Significant difference (Y/N)	N	Y for collagenase- and explant-derived	N
Clonogenic potential	Higher in trochlear samples in young animals	Decreases with age	Lower in explant-derived cultures
Significant difference (Y/N)	Y for collagenase-derived in young	Y	Y
Matrix synthesis (Size of pellets)	Higher in trochlear samples in young animals	Reduces with age	No effect
Significant difference (Y/N)	Y in collagenase- and explant-derived in young	Y	N
Chondrogenic potential	High inter-individual variability; Higher in upper trochlea in young animals	Decreases with age	No effect
Significant difference (Y/N)	Y	Y	N
Osteogenic potential	Higher in upper trochlea in young animals	Decreases with age	No effect
Significant difference (Y/N)	Y for collagenase and explant-derived in young	Y	N

4.4 Discussion

Clear biological differences were observed in biological properties of BMPCs from trochlea and condyles, with trochlear cells-more specifically trochlea upper cells-exhibiting increased cell yield, more clonogenic potential and differentiation potency, thus confirming our first hypothesis that location influence chondrogenic potential. Since P0 cell yields reflect *in situ* difference in initial number of progenitor cells present at these two sites, we can conclude that fewer chondrogenic progenitors reside in condyle compartment versus trochlea. Close interaction of densely-packed cells during condensation phase is critical for initiation of chondrogenic differentiation of BMPCs-since high number of cells are needed in initial stages- and a low density BMPC population in condyles could be directly correlated to poor repair outcome. Also, since the number of self-renewing cells possessing clonogenic potential is positively correlated with repair potential, lower CFU-f might reduce *in vitro* chondrogenic potential of condyles. At molecular level, a difference in the expression of Sox-9 -master regulator of chondrogenic determination of BMPCs [15] or response of BMPCs to TGFβ-III might be yet another factor responsible for poor chondrogenic differentiation with condylar BMPCs. Although MSCs niche is deemed to be dynamic, it is challenging to determine their mobilization and *in situ* behaviour in the absence of methods for their localization *in vivo*. As is common in *in vivo* studies, inter-animal variation was also prominent in pellet cultures in young animals. Medium- and low-responders showed significantly improved cartilaginous phenotype in trochlear pellets indicated by higher GAG and collagen type II expression, consistent with our earlier observations of better repair outcome in rabbits [3, 4]. Alternatively, high-responders consistently generated matrix rich in cartilage components in all samples. However, it is impossible to ascertain if this observation was due to inherent differences or influence of *in vitro* culture conditions on differentiation potency. For instance, the same number of condylar and trochlear BMPCs were used for pellet culture. *In vivo*, the number of cells participating in cartilage repair would depend on initial population of BMPCs which are scarcer in condyles, based on our cell yield assay. Moreover, an ideal and identical cocktail of growth factors and other cytokines was provided to BMPCs *in vitro*, unlike *in vivo*. Previous studies pointed out that TGF and BMPs released from the underlying subchondral bone influence cell migration and repair tissue formation [16, 17]. *In vivo* differences in production, release, and metabolism of these cytokines and responses from condyle vs trochlea would be less pronounced *in vitro*. MSC populations are known to be heterogeneous

mixture of uni-, bi- and tripotent subpopulations with varying levels of lineage commitment [20]. It is possible that a population isolated from a particular donor is richer in bi- and tripotential progenitors and might have higher chondrogenic potential. This might explain higher chondrogenesis seen in some condylar high-responders since even condylar BMPCs with low yield but relatively higher proportion of chondroprogenitors might have a superior differentiation potential. Finally, a clear influence of location was observed when cells were stimulated for osteogenic differentiation. Although, mineralization appeared comparable upon macroscopic examination, quantitative assay indicated a weaker osteogenic phenotype of condylar cells. Due to its role in cartilage repair, better subchondral bone repair in trochlea might positively influence the cartilage repair. It also re-emphasizes the superior differential potential of trochlea. Interestingly we did not observe any influence of location on expression of cell surface markers. Although used frequently for characterization, these markers are not specific MPC markers and do not indicate their levels of self-renewal or differentiation capacity.

In our previous animal studies, cartilage repair outcome was superior when subchondral bone was drilled to 6mm compared to 2mm [6] motivating us to examine BMPCs isolated from epiphyseal (upper) and metaphyseal (lower) marrow. In this study we found yield and differentiation potential to be superior in trochlea upper versus lower, leading us to reject our hypothesis that more progenitors occur in the deeper metaphyseal marrow. It is important to consider here that volume of subchondral bone participating in repair in deep drilling is three times that of shallow drilling and therefore produces a larger volume of GAG+ callus [6]. In young animals, such a large callus could have potential to proliferate towards the articular cavity and produce articular repair cartilage tissue, while in older animals; callus with continued proliferation of senescent cells could lead to an outgrowth of fibroblasts into the articular cavity leading to a fibrous repair. One limitation of this study is that recruitment of progenitor cells in the clinical setting may be different than how cells were isolated here *in vitro* (collagenase or explant culture).

Increasing age had a profound impact on all the biological properties studied here confirming our second hypothesis that age influences chondrogenic potential was true. Our data supported the earlier findings which report the reduction in differentiation potency of stem cells in older animals [18]. Older animals were characterized by reduced number as well clonogenic potential in addition to a weaker chondrogenic and osteogenic differentiation potential. CFU-f has been

reported to be inversely correlated to age leading to a decrease in differentiation potential in senescent animals [19]. Moreover, frequency of tripotential clones reduces with increasing age- replaced first by bipotential and then unipotential clones- thus reducing differentiation potential [20]. Studies indicate reduction in pellet size with increasing age [21] and poor expression of Col-II accompanied by negligent GAGs expression in older rats [22]. BMPCs possess very low levels of telomerase-enzyme responsible for maintaining telomere length [23]. In addition, there is a downregulation of genes involved in DNA replication, cell cycle, mitosis and DNA repair with age [24]. Most importantly, expression of stemness related markers including Oct-4, Sox-2 and Nanog has also been found to diminish with increasing age [25]. Taken together, the past knowledge combined with the observations in this study may be used in development of approaches to overcome this deleterious effect of age on stem cells and improve the repair outcome in clinical situations.

Our third hypotheses-that biological properties of BMPCs are unaffected by method of isolation- was partially supported, given that explant-derived and collagenase-derived cultures had similar differentiation potential though there were some striking differences in the clonogenic potential. Age had a more profound impact on the yield of progenitor cells derived from bone since the yield of explant-derived BMPCs was comparable to collagenase-derived cultures in young animal while reduced drastically in older animals. Bone chips from young rabbit still possess growth plate with on-going endochondral ossification. This could influence the type of cells growing out of the explant in young (chondrocytes and osteocytes) vs. old (osteocytes only). This heterogeneity might explain the reduced chondrogenic differentiation potential of explant derived BMPCs in old animals. In addition, decline in progenitor cell population or reduced migration potential of existing progenitors might lead to poor BMPC yield in explants from older animals. Clonogenic potential was found to be particularly depleted in explant-derived cultures which could be a function of both method of isolation and culture conditions. Moreover, since explant-derived BMPCs are believed to originate from de-differentiation of osteoblast cells [8], it is possible that population of such cells is very low in the initial passage used for CFU-f assay. The homogeneity of cultures along with an increase in population of undifferentiated MPCs might lead to an improved chondrogenic and osteogenic differentiation potential comparable to collagenase-derived cultures in later passages. Cells recovered from old explants appear to be

senescent or to have lost TGF receptor expression or chondrogenic capacity, and may be only capable of generating fibrous tissue. This result could be related to the fact that young bone is undergoing remodeling and therefore probably contains progenitor cells embedded in the collagen type I matrix; inaccessible to collagenase. Moreover, in explant cultures, trace residual collagenase could more readily liberate cells from partly mineralized young bone matrix than heavily mineralized old bone. Along these lines, we speculate that remodeling bone could potentially liberate stem cells into the repair zone. Our findings are also supported by a recent study which reported comparable *in vitro* biological properties including proliferation and differentiation potential for collagenase- and explant-derived BMPCs isolated from several tissue sources [26]. In summary, despite differences in the initial passages, the origin of cells from explant versus collagenase-derived does not affect the differentiation potential of cultures and therefore is not expected to influence the *in vivo* cartilage repair outcome.

4.5 Conclusion

This study is first of its kind to carry out detailed analysis of biological properties of young and old BMPCs isolated from two locations of knee using cells obtained with two different isolation methods. Location of BMPCs was found to be an important factor influencing yield, clonogenicity and differentiation potential of BMPCs. A wide variation was observed in chondrogenic potential in younger animals, where some young animals showed the same loss of capacity for proliferation, stemness and potency as BMPCs isolated from older animals. The mechanisms to explain young non-responders remain elusive. Finally, biological properties-with an exception of clonogenic potential- were similar for explant and collagenase-derived cells. Owing to high spontaneous repair potential of young animals and limitations of *in vitro* models in simulating *in vivo* conditions, a study which directly correlates the properties of stem cells and repair outcome in an animal model might be the next step in understanding pre-existing differences at the two locations. Taken together, our data suggests an influence of location as well as age on the *in vitro* biological performance of BMPCs that could be directly responsible for location and age-dependent repair outcome seen in our previous animal studies. These results enhance our understanding location and age-dependent cartilage repair in humans and point towards effective approaches to improve repair outcomes, for example by stimulating cell

recruitment from subchondral sites via surgical techniques and biological factors as well as promoting subsequent proliferative and specific differentiation behaviours.

Acknowledgements

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CHAPTER 5 ARTICLE 2: QUALITY OF CARTILAGE REPAIR FROM MARROW STIMULATION CORRELATES WITH CELL NUMBER, CLONOGENIC, CHONDROGENIC AND MATRIX PRODUCTION POTENTIAL OF UNDERLYING BONE MARROW STROMAL CELLS

The previous study provided tangible evidence of influence of location and age on the properties of condylar and trochlear BMPCs/bone marrow stem cells (MSCs). Earlier studies have reported location-dependent variability in marrow stimulated repair outcome in multiple animal studies. However, correlation of repair outcome and properties of MSCs has never been reported before. The following chapter presents the first study designed to correlate the properties of subchondral MSCs with location dependent variability in the marrow stimulated cartilage repair. Defects were created on condyle and trochlea in one knee of skeletally mature rabbits and followed by treatment with BMS. Qualitative assessment of three week repair tissue was carried out to assess the repair outcome in condyle and trochlea. Collagenase and explant cultures of MSCs were derived from condyle and trochlea of contralateral knee. MSCs were characterized to determine the influence of location and donor on their number, clonogenicity and differentiation potential. Statistical analysis enabled us to determine the factors with maximum impact on the repair outcome likely revealing a mechanism of variability observed in BMS. Correlation analysis was applied to determine if location dependent variability in cartilage repair responses stem from inherent differences in the biological properties of subchondral MSCs including their number and differentiation potential. We believe that these observations will eventually contribute towards improving the outcome of BMS procedure and enable clinicians to develop it in a more patient relevant basis to maximize its efficiency. *Submitted-Osteoarthritis and Cartilage*.

QUALITY OF CARTILAGE REPAIR FROM MARROW STIMULATION CORRELATES WITH CELL NUMBER, CLONOGENIC, CHONDROGENIC AND MATRIX PRODUCTION POTENTIAL OF UNDERLYING BONE MARROW STROMAL CELLS

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ABSTRACT

Introduction: Variable cartilage repair outcomes following bone marrow stimulation for articular cartilage repair suggest that subchondral bone marrow stem cells (BMSCs) in different donors and locations have different intrinsic capacity for cartilage repair. Using a rabbit model, we tested the hypothesis that *in vivo* cartilage repair correlates with *in vitro* biological properties of BMSCs.

Methods: Full thickness cartilage defects were created in the trochlea and condyle in one knee of skeletally mature New Zealand White rabbits (N=8) followed by microdrilling. Three week repair tissues were analyzed by macroscopic ICRS scores, O'Driscoll histological scores and Safranin-O (Saf-O) and type-II collagen (Coll-II) % stain. BMSCs isolated from contralateral knees were assessed for cell yield, surface marker expression, CFU-f, %Saf-O and %Coll-II in pellet culture followed by correlation analyses with the above cartilage repair responses.

Results: *In vivo* cartilage repair scores showed strong, positive correlation with *in vitro* cell yield, clonogenic potential, and chondrogenic matrix production (Coll-II, GAG) by TGF-βIII stimulated BMSC cultures. Trochlear repair showed clear evidence of donor dependency and strong correlation was observed for inter-donor variation in repair and the above *in vitro* properties of trochlear BMSCs. Correlation analyses indicated that donor- and location-dependent variability observed in cartilage repair can be attributed to variation in the properties of BMSCs in underlying subchondral bone.

Conclusion: Variation in cell number, clonogenic, chondrogenic and matrix production potential of BMSCs correlated with repair response observed *in vivo* and appear to be responsible for inter animal variability as well as location-dependent repair.

5.1 Introduction

Chondral lesions are a commonly occurring knee pathology and are more widespread in medial femoral condyle (MFC) than trochlea [1]. Bone marrow stimulation (BMS) procedures carried out by fracturing or drilling into subchondral bone typically produce a fibrocartilaginous repair. Repair outcomes are affected by defect location suggesting a strong influence of biological characteristics of progenitor cells in the underlying subchondral bone on repair response [2, 3]. Donor-, location- and age-related factors are increasingly being recognized to influence repair outcomes and may guide patient-specific treatment [4]. The mechanisms behind these sources of variable cartilage repair still elude us since underlying factors such as bone structure and mechanics, load-bearing condition and role of subchondral bone progenitor cells are largely unknown.

Better repair and increased chondrogenic potential have been observed in rabbit trochlea compared to medial femoral condyle in previously published studies [5, 6]. BMS cartilage repair in rabbit trochlea was associated with more chondrocytes and larger chondrogenic foci suggesting greater chondrogenic potential of trochlear progenitor cells compared to condylar progenitors [5]. As evidence of location- and species-dependent cartilage repair, more fibrous repair was observed in ovine trochlea versus condyle with BMS as well as with autologous chondrocyte implantation [7, 8]. *In vitro* characterization of BMSCs isolated from condyle and trochlea of young and old rabbits showed evidence that trochlear BMSCs were more numerous with higher clonogenic and chondrogenic differentiation potential [9]. Since BMS relies on recruitment of underlying BMSCs for repair of soft and hard tissues, characterization of these progenitors and direct correlation with cartilage repair outcomes may provide important mechanistic insights into location-dependent cartilage repair.

Here, in addition to validating the influence of location on early repair responses in an acute defect rabbit model, we sought to investigate the influence of biological properties of BMSCs isolated from condyles and trochlea on the repair response. Although repair responses induced by BMS along with characterization of BMSCs has been investigated in animal models before [10-13], this is the first study designed to correlate the early repair response with biological properties of BMSCs from the same location to elucidate the mechanism of location-dependent

and inter-animal variation observed in cartilage repair. Since cells participating in marrow-stimulated cartilage repair originate from both stromal spaces and bone lining regions, BMSCs were isolated from both these regions by means of collagenase digestion and cell outgrowth from explants, respectively. Based on our earlier observations, we do not expect any significant difference in the properties of cells isolated by these methods of isolation [9]. The current study was carried out with the hypothesis that the quality of cartilage repair outcome in condyle and trochlea will correlate to the *in vitro* biological properties of underlying BMSCs at the same two sites in the contralateral knee.

5.2 Materials and methods

5.2.1 Study design and rabbit surgical model

The research protocol was reviewed and approved by an institutional ethics committee for animal research. Eight skeletally mature (8-9 month old) female New Zealand White rabbits were used. In order to examine the influence of inter-animal variation, a bilateral model was used for characterization of cartilage repair and *in vitro* biological properties of BMSCs [Fig.5.1]. On one knee (n=8 knees), full thickness cartilage defects measuring 4 X 4 mm were created on the medial femoral condyle (MFC) and central trochlear groove by complete debridement of non-calcified and calcified cartilage using a flat blade to expose the underlying subchondral bone. Using drilling burrs, four subchondral drill holes, 0.9 mm diameter and 6 mm deep, were made in each defect in the trochlea and MFC [14]. Constant cooling irrigation was applied to prevent heat necrosis [14] and remove loose bone debris. The patella was repositioned and the knee was closed in sutured layers. No peri-operative antibiotics were administered but animals received extended analgesia with a fentanyl transdermal patch. Knees were allowed unrestricted motion and constantly monitored for infections and other complications until sacrifice three weeks later. The contralateral knee (n=8 knees) in each animal was intended for *in vitro* characterization of BMSCs from condyle and trochlea and received no treatment prior to sacrifice [Fig. 5.1].

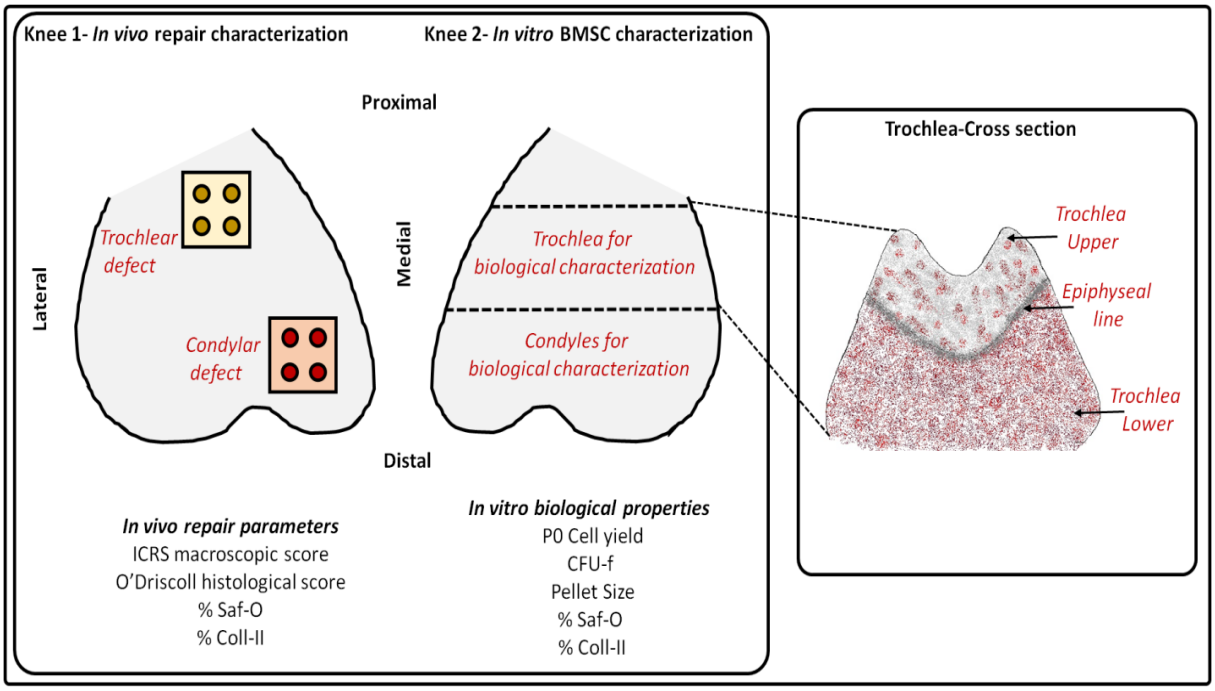

Figure 5.1: Schematic representing the study design. Condylar and trochlear cartilage repair outcome in one knee from each animal was correlated with *in vitro* biological properties of BMSCs isolated from the condyle, upper trochlea and lower trochlea from the contralateral knee.

5.2.2 Characterization of cartilage repair

Animals were sacrificed by overdose of pentobarbital and the treated knee was characterized for cartilage repair. Photographs of harvested joints were scored using the macroscopic ICRS scoring system for cartilage repair [15]. Scoring was performed by two blinded readers (ICC 0.95 for overall repair assessment). Decalcified samples were embedded in OCT and sections were obtained from three levels-through proximal and distal holes in addition to between holes. Sections were stained with Safranin O/Fast Green [16] and scanned using a Nanozoomer RS system (Hamamatsu, Japan). Two blinded observers scored the digital Saf-O stained sections using a previously published modified O'Driscoll histological scoring method (ICC 0.86 for total O'Driscoll score) [13]. The average score from two observers was used to determine final macroscopic ICRS and O'Driscoll score. All three sections collected from each defect were also

used to determine %Saf-O and %Coll-II in the chondral repair tissue using a previously described method of Hue-Saturation-Value thresholding with ImageJ and in-house Matlab routine software [16].

5.2.3 Isolation and *in vitro* characterization of condylar and trochlear BMSCs

The contralateral knee was used for isolation of BMSCs as previously described [9]. Briefly, distal femur ends were separated into condyles, trochlea upper and trochlea lower compartments [Fig.5.1] followed by fragmentation into bone chips (approx. 5mm) which were then digested with collagenase type XI (Sigma) [3mg/ml, 2hours] to obtain the population of collagenase-derived cell BMSCs. Digested bone chips were then seeded as explants to generate a second population of explant- derived cell BMSCs. BMSCs were cultured for a maximum of 2 passages in DMEM/F-12 media supplemented with 10% fetal bovine serum and 1X penicillin-streptomycin. The following biological properties were then characterized: P0 cell yield, CFU-f, cell surface marker expression and chondrogenic differentiation potential using previously reported methods [9]. P0 cell yield was used to estimate the population density of BMSCs originally present in condyles and trochlea. Stemness of trochlear and condylar BMSCs was characterized by CFU-f assay. Cell surface marker characterization was done for positive - CD44 (Cell Marque, 144M-96) and CD29 (Millipore, MAB1951Z) and negative - CD34 (Cell Marque, 134M-16) and CD45 (Cell Marque, 145M-97) stem cell markers [9]. BMSC pellets were used for chondrogenic differentiation for 3 weeks in a 15 mL conical tube in chondrogenic medium comprised of high glucose DMEM containing penicillin-streptomycin, ascorbate, dexamethasone, sodium pyruvate, BSA and TGFβ-III [9]. Size of pellets was measured to determine the amount of matrix synthesized as an indicator of chondrogenic differentiation. Safranin-O/fast green staining and collagen-type II immunostaining was done as described previously. Stained sections of pellets were used to determine the percentage area stained positively for Saf-O (% Saf-O_pellets) and Collagen-II (% Coll-II_pellets) using Image J and a Matlab routine as described above.

5.2.4 Correlation of cartilage repair to BMSC properties

Correlations between five *in vitro* assessed biological properties of BMSCs (P0 cell yield, CFU-f, Pellet Size (mm), %Saf-O + and %Coll-II + area in pellets) and four *in vivo*

assessed repair parameters (ICRS Score, O'Driscoll Score, %Saf-O + and %Coll-II + repair tissue) was carried out for collagenase- and explant-derived BMSCs.

5.2.5 Statistical Analyses

Statistical analysis was performed using SAS Enterprise Guide 5.1 and SAS 9.3. Since several segments were collected from both legs of each rabbit, a mixed model was used to account for the influence of donor. Fixed effects were location (condyles, upper trochlea or lower trochlea for *in vitro* characterization, n=8 knees and condyles and trochlea for cartilage repair characterization, n=8 knees) while donor was a random effect. Data in figures are presented as mean (diamond); median (line); Box: 25^{th} and 75^{th} percentile; Whiskers: Box to the most extreme point within 1.5 interquartile range. Correlations between *in vitro* BMSC properties and *in vivo* repair outcome were analyzed by calculating the Pearson correlation coefficients. $p<0.05$ were considered statistically significant. In order to determine the *in vitro* variables which best account for the variability in repair outcome, a general linear model (GLM) was applied. For each *in vivo* repair parameter, variables with the least influence (highest value of p) were sequentially removed in a step wise manner to yield variables with the most significant influence ($p<0.05$) on repair outcome.

5.3 Results

5.3.1 Repair of trochlear defects was superior to condylar defects- and correlated with *in vitro* properties of BMSCs as function of location and donor

We focussed on the early events in cartilage repair and found that quality and quantity of condylar repair tissue was markedly inferior in contrast to mostly excellent trochlear healing. At 3 weeks post-surgery, six out of eight trochlear defects were filled with glossy, white and smooth repair tissue flush with adjacent cartilage [Fig. 5.2a]. These donors were termed good trochlear responders. The majority of these repair tissues were homogenous with a smooth surface and appeared to bond well with adjacent tissue with occasional cases heterogeneous with slightly irregular surface and identifiable defect margins. Defects were significantly depressed and incompletely filled in two donors leading us to identify them as poor trochlear responders [Fig. 5.2b]. In contrast, all eight condylar defects were severely depressed with very little fill in addition to distinct residual drill holes and defect margins [Fig 5.2c, d]. Mean ICRS macroscopic scores were significantly higher for trochlea versus condyles (p=0.0027) [Fig. 5.2e]. Moreover, ICRS grade II (nearly normal) was assigned to 5 out of 8 trochlear repair tissues while all 8 condylar repair tissues were scored ICRS grade IV (severely abnormal).

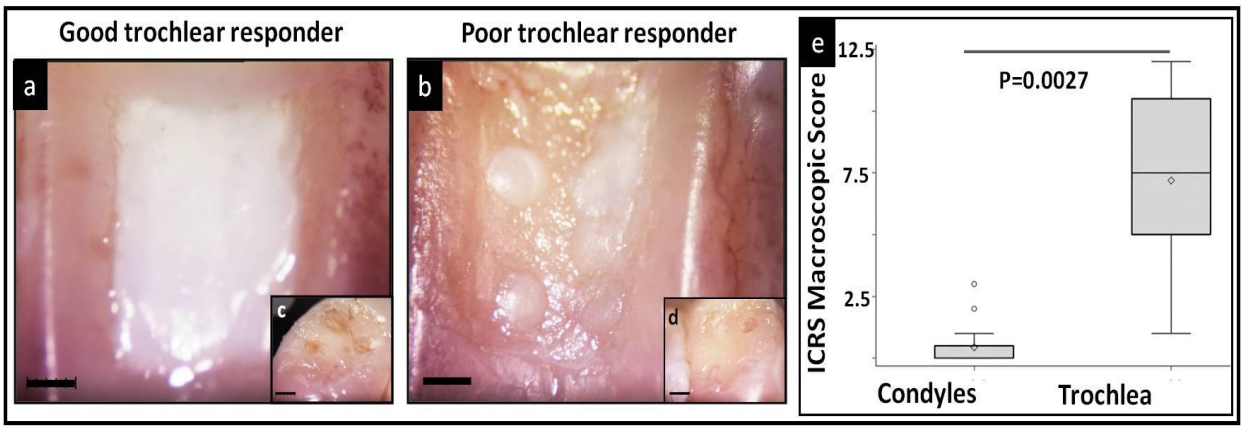

Figure 5.2: Macroscopic appearance of repair tissue in good and poor trochlear responders at 3 weeks post-operative. Acute trochlear defects treated with marrow stimulation were largely restored with repair tissue in six of eight donors termed good responders (a) while two donors had extremely poor repair outcome and were termed poor responders (b). Condylar defects were incompletely filled with distinct holes and defect margins in all 8 donors (inserts c,d are from

corresponding condylar repair outcomes in good and poor trochlear responders). Scale bar=1mm. Significantly higher mean macroscopic ICRS scores indicate superior early repair response in trochlea versus condyles (e).

Saf-O stained sections of condyles showed thin, fibrous repair tissue with poor structural integrity in contrast to voluminous tissue with regular surface observed in trochlear defects [Fig. 5.3a-h]. Average O'Driscoll scores were significantly reduced for condylar repair tissues versus trochlea ($p<0.0001$) [Fig. 5.3i] and associated with lower scores for surface regularity, thickness of repair tissue, cell morphology, health of adjacent cartilage and negligible GAG accumulation. Upon quantitative histomorphometric analysis, significantly reduced % Saf-O ($p=0.002$) [Fig. 5.3j] and % Coll-II ($p=0.0048$) [Fig. 5.4i] were observed for condyles.

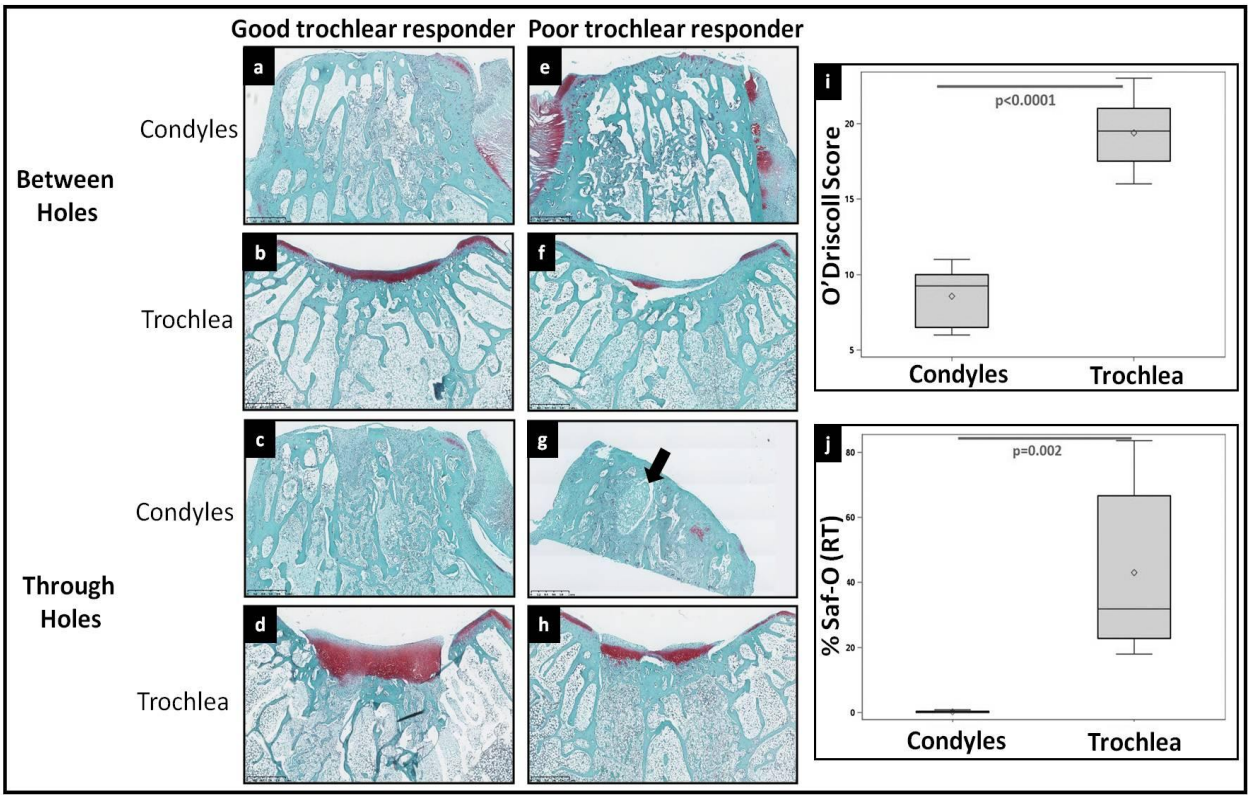

Figure 5.3: Representative Saf-O/Fast Green (a-h) staining of repair response in sections collected between holes (a,b,e,f) and through holes (c,d,g,h) in good (a-d) and poor (e-f) trochlear responders. Depleted Saf-O staining was evident in all condyles. Among good trochlear responders, abundant GAG expression was observed, more evident in sections from between

holes. Expression was reduced in poor trochlear responders. Arrow points to subchondral cyst observed in the condyle of a poor responder. Scale bar=1mm. Significantly higher O'Driscoll scores indicate superior early repair response in trochlea versus condyles (i). Trochlear matrix was more abundant in GAG indicated by significantly higher %Saf-O (j) in repair tissues

Clear histological differences were observed between good and poor trochlear responders *in vivo*. In the sections taken from between holes, thicker repair tissue with abundant GAG was observed in good trochlear responders in contrast to thin, fibrous tissues in poor trochlear responders with negligible GAG expression. In trochlear repair tissue through the microdrill holes, Saf-O staining was observed in mid-deep regions of good trochlear responders with varied levels of hyaline and fibrocartilagenous tissue. In contrast, repair tissue was thinner and more fibrous in poor responders indicated by diminished Saf-O (Avg. %Saf-O-49.46$\pm$13.72 in good responders vs 26.62$\pm$8.1 in poor responders) [Fig 5.3b vs f and 5.3d vs h]. The only instance of an osteochondral cyst was observed in the condyle of one of the poor responders [Fig. 5.3g]. Trochlear repair tissues also expressed greater levels of collagen type II compared to condyles [Fig. 5.4a-h]. Among trochlea, Coll-II was more widespread in good responders while poor responders showed evidence of depleted Coll-II staining (Avg. % Coll-II -56.67$\pm$23.64% in good responders vs 20.6$\pm$2.29 in poor responders) [Fig. 5.4].

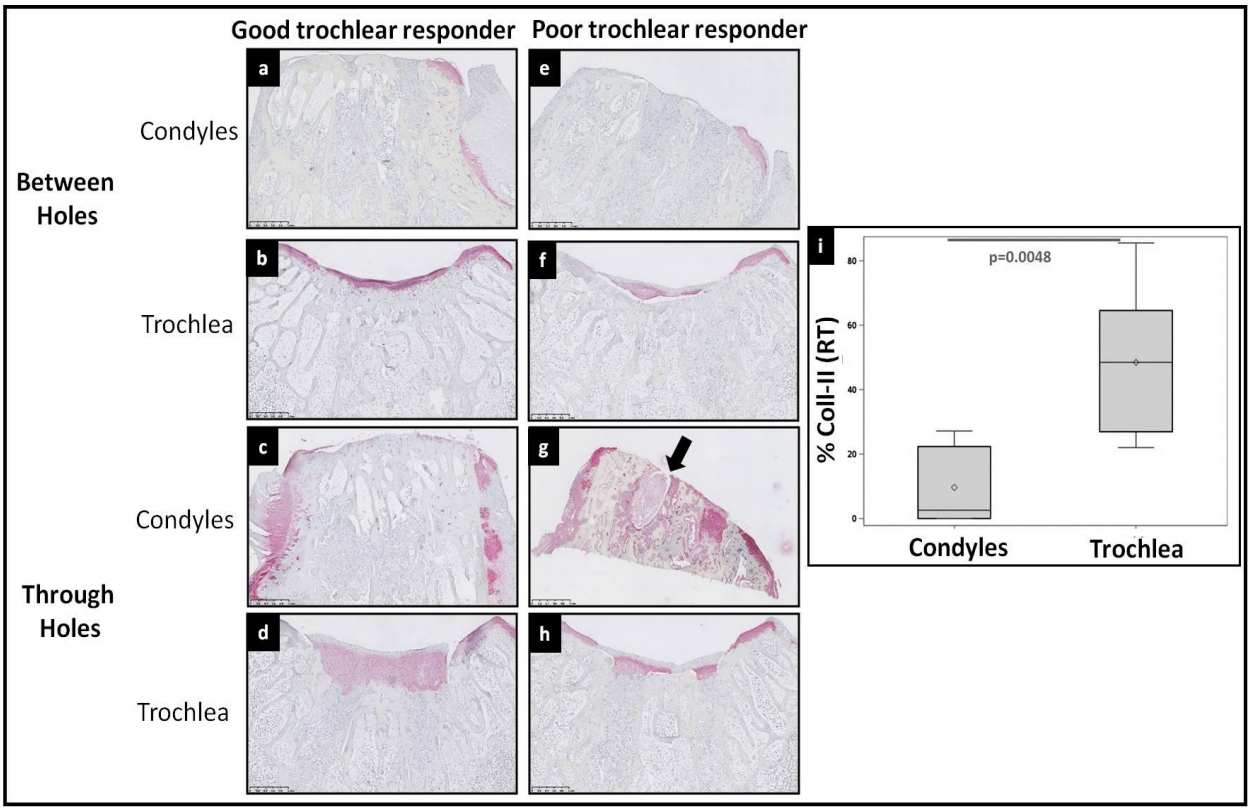

Figure 5.4: Representative Coll-II (a-h) staining of repair response in sections collected between holes (a,b,e,f) and through holes (c,d,g,h) in good (a-d) and poor (e-h) trochlear responders. Reduced Coll-II staining was observed in all condyles. Increased collagen expression was observed in good vs poor trochlear responders, more evident in sections taken from between holes. The arrow points to a subchondral cyst observed in the condyle of poor responder. Scale bar=1mm. Trochlear matrix was more abundant in collagen type II indicated by significantly higher % Coll-II (i) in repair tissues.

Pellets cultured without TGFβ-III were distinctly smaller than TGFβ-III-stimulated cultures for both collagenase- and explant-derived BMSCs. Among the pellets stimulated with TGFβ-III, trochlea upper pellets had the largest diameter while condyles generated the smallest pellets for both collagenase- ($p<0.0001$) and explant-derived BMSCs ($p=0.0013$) [Fig. 5.5, 5.1S c,h; 5.2S-b]. In general, trochlear pellets had increased GAG and Coll-II in collagenase- and explant-derived BMSCs [Fig. 5.5]. Interestingly, trochlea lower pellets were smaller in poor trochlear responders for both collagenase- and explant-derived BMSCs [Fig. 5.5 and 5.2S]. Trochlea

upper pellets were associated with abundant GAG and coll-II in good as well as poor trochlear responders while trochlea lower pellets from poor trochlear responders had reduced Saf-O staining indicating low GAG deposition [Fig. 5.5]. As a further evidence of poor chondrogenic differentiation potential in cells derived from condylar subchondral bone, the matrix of condylar pellets was depleted in GAG and Coll-II as indicated by lower % Saf-O [Fig. 5.1S d,i] and % Coll-II [Fig. 5.1S e,j]. Trochlear pellet matrix had higher %Saf-O (collagenase-derived BMSCs, $p<0.0001$ and explant-derived BMSCs, $p=0.0009$) and %Coll-II versus condylar pellets (collagenase-derived BMSCs, $p<0.0001$ and explant-derived BMSCs $p=0.0002$).

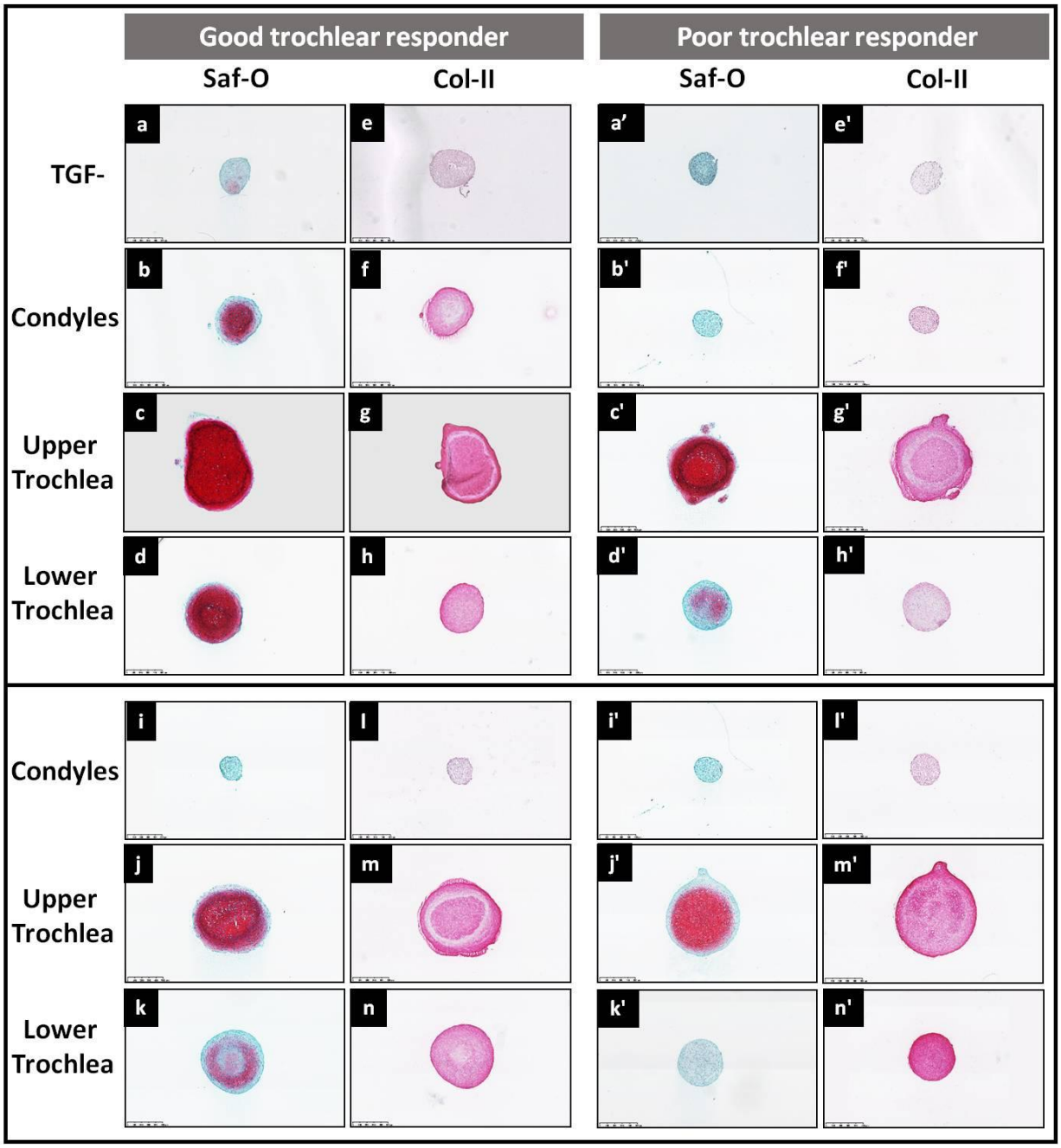

Figure 5.5: Safranin-O/fast-green and collagen type II staining of pellets from good [a-n] and poor [a'-n'] trochlear responders. Negligible GAG and collagen type II expression was observed in condyles indicating inferior chondrogenic potential *in vitro*. GAG was low in trochlea lower pellets in trochlea poor responders. a-h, a'-h' are collagenase-derived; i-n, i'-n' are explant-derived BMSCs. TGF- are from upper trochlea, while all other cultures had TGFβ-III. Scale bar=500 µm

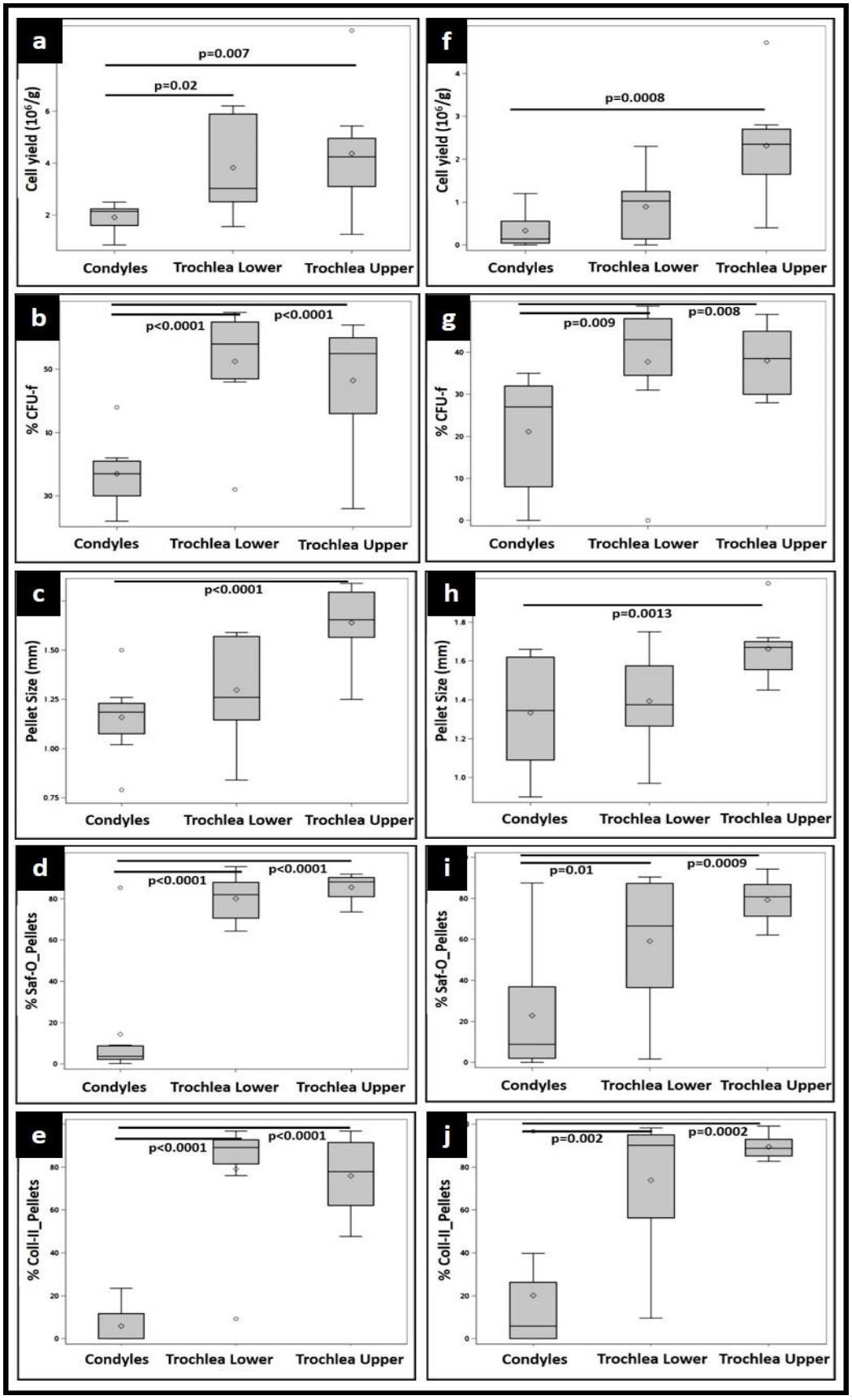
a
p=0.007
p=0.02
Cell yield (10^6/g)
Condyles
Trochlea Lower
Trochlea Upper
f
p=0.0008
Cell yield (10^6/g)
Condyles
Trochlea Lower
Trochlea Upper
b
p<0.0001
p<0.0001
% CFU-f
Condyles
Trochlea Lower
Trochlea Upper
g
p=0.009
p=0.008
% CFU-f
Condyles
Trochlea Lower
Trochlea Upper
c
p<0.0001
Pellet Size (mm)
Condyles
Trochlea Lower
Trochlea Upper
h
p=0.0013
Pellet Size (mm)
Condyles
Trochlea Lower
Trochlea Upper
d
p<0.0001
p<0.0001
% Saf-O_Pellets
Condyles
Trochlea Lower
Trochlea Upper
i
p=0.01
p=0.0009
% Saf-O_Pellets
Condyles
Trochlea Lower
Trochlea Upper
e
p<0.0001
p<0.0001
% Coll-II_Pellets
Condyles
Trochlea Lower
Trochlea Upper
j
p=0.002
p=0.0002
% Coll-II_Pellets
Condyles
Trochlea Lower
Trochlea Upper

Figure 5.1S: Summary of in vitro biological properties of collagenase- (a-e) and explant-derived BMSCs (f-j). Higher values were observed for trochlea versus condyles for following properties -P0 cell yield (a,f); CFU-f (b,g); pellet size (c, h); % Saf-O_Pellets (d,i) and % Coll-II_Pellets (e,j).

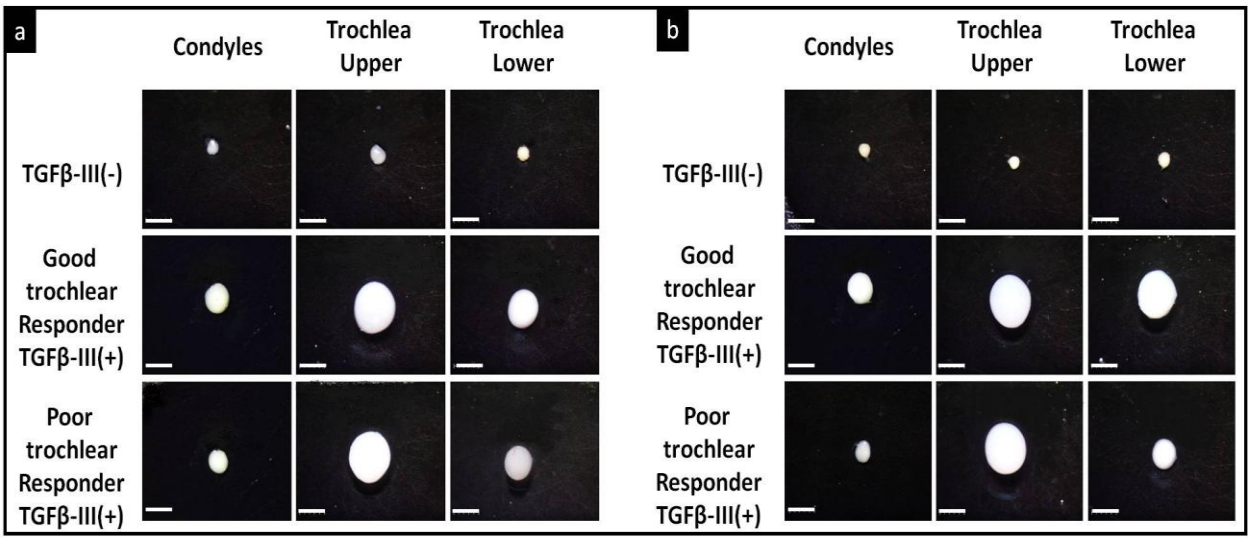

Figure 5.2S: Gross structure of pellets show an increase in size upon culture in presence of TGFβ-III. Pellets from trochlea upper were largest compared to condyles in good and poor trochlear responders. Trochlea lower pellets were smaller in poor trochlear responders. a). collagenase-derived; b). explant-derived BMSCs. Scale bar=1 mm

5.3.2 Cell yield, clonogenic potential and expression of stem cell markers are higher in trochlear vs condylar BMSCs and these properties decreased in poor trochlear responders

In vitro biological properties were found to be influenced by location as well as donor. In comparison to condyles, higher cell yield was observed in trochlea for both collagenase- ($p=0.007$) and explant-derived BMSCs ($p=0.0008$) [Fig. 5.1S a,f]. BMSCs from trochlear segments also had higher clonogenic potential indicated by higher CFU-f for both collagenase ($p<0.0001$) and explant-derived BMSCs ($p=0.008$) [Fig. 5.1S b,g]. In contrast, the two poor trochlea responders showed a noticeable reduction in cell yield and CFU-f in both trochlea upper and trochlea lower [Table 5.1S].

Table 5.1S: Summary of average cell yield and CFU-f from good and poor trochlear responders for collagenase- and explant-derived BMSCs, highlighting the difference between good and poor trochlear responders.

	Collagenase-derived		Explant-derived	
Responder	Trochlea Upper	Trochlea Lower	Trochlea Upper	Trochlea Lower
	Cell yield (X10E6/g)		Cell yield (X10E6/g)	
Good	5.25 ± 1.8	4.45 ± 1.7	2.3 ± 1.4	0.97 ± 0.07
Poor	1.73 ± 0.66	1.93 ± 0.52	2.3 ± 0.6	0.6 ± .07
	CFU-f (%)		CFU-f (%)	
Good	53.2 ± 3.4	55.2 ± 3.8	41.2 ± 6.6	38 ± 3.6
Poor	33.4 ± 7.7	39.5 ± 12.1	28 ± 0.7	34 ± 4.9

Expression of cell surface markers was similar between condyles and trochlea. However, expression varied as a function of donor and was found to be weaker for collagenase- [Fig. 5.3S a,b] as well as explant-derived BMSCs [Fig. 5.3S e,f] in both poor trochlear responders. This reduced expression was only observed for CD44 and not CD29 (data not shown) - the other stem cell marker analyzed. Expression of CD34 and CD45 was absent for all donors for collagenase- and explant-derived BMSCs indicating absence of any contamination by cells of hematopoetic origin [Fig. 5.3S].

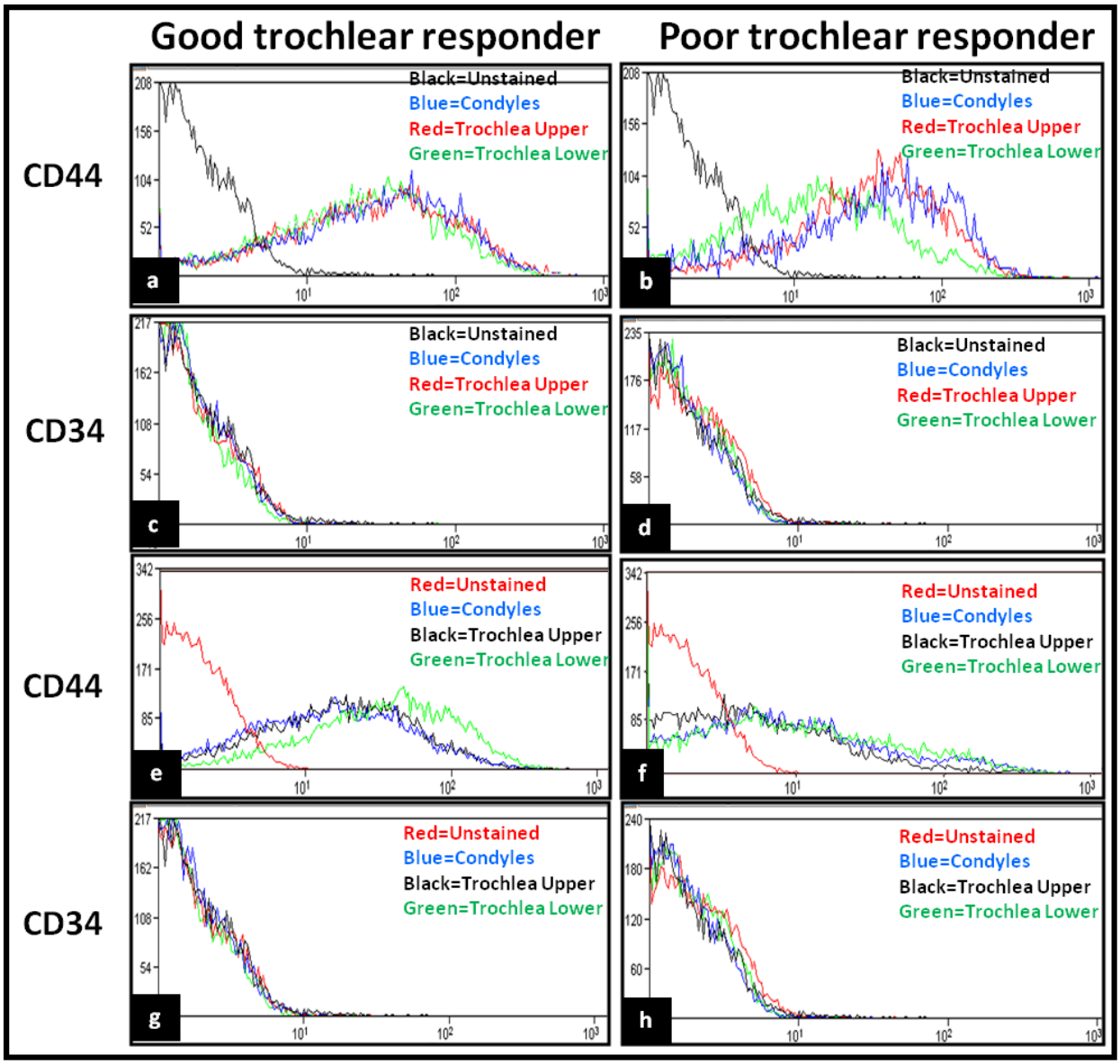

Figure 5.3S: CD 44 expression was reduced in trochlea lower in collagenase-derived BMSCs (a vs b) and in all samples in explant-derived BMSCs (e vs f). CD 34 expression was consistently absent (c,d,g,h).

5.3.3 Moderate to strong positive correlations were observed between *in vitro* properties of collagenase-derived BMSCs and *in vivo* early repair responses and strong influence of cell yield, CFU, % Coll-II was observed

Significant correlations were found between *in vitro* BMSC properties and early repair response in condyle and trochlea. Robust positive correlations between cell yield, CFU-f and % Coll-II (pellets) of collagenase-derived BMSCs and all *in vivo* repair properties studied [Fig 5.6, 5.7] indicated a strong influence of these BMSCs properties on cartilage repair outcome (r^2 values ranged from 0.48 to 0.73). Pellet size and % Saf-O (pellets) showed significant, positive but modest correlation with most of the *in vivo* repair parameters studied (r^2 values ranged from 0.26 to 0.44) [Figs. 5.6 and 5.7].

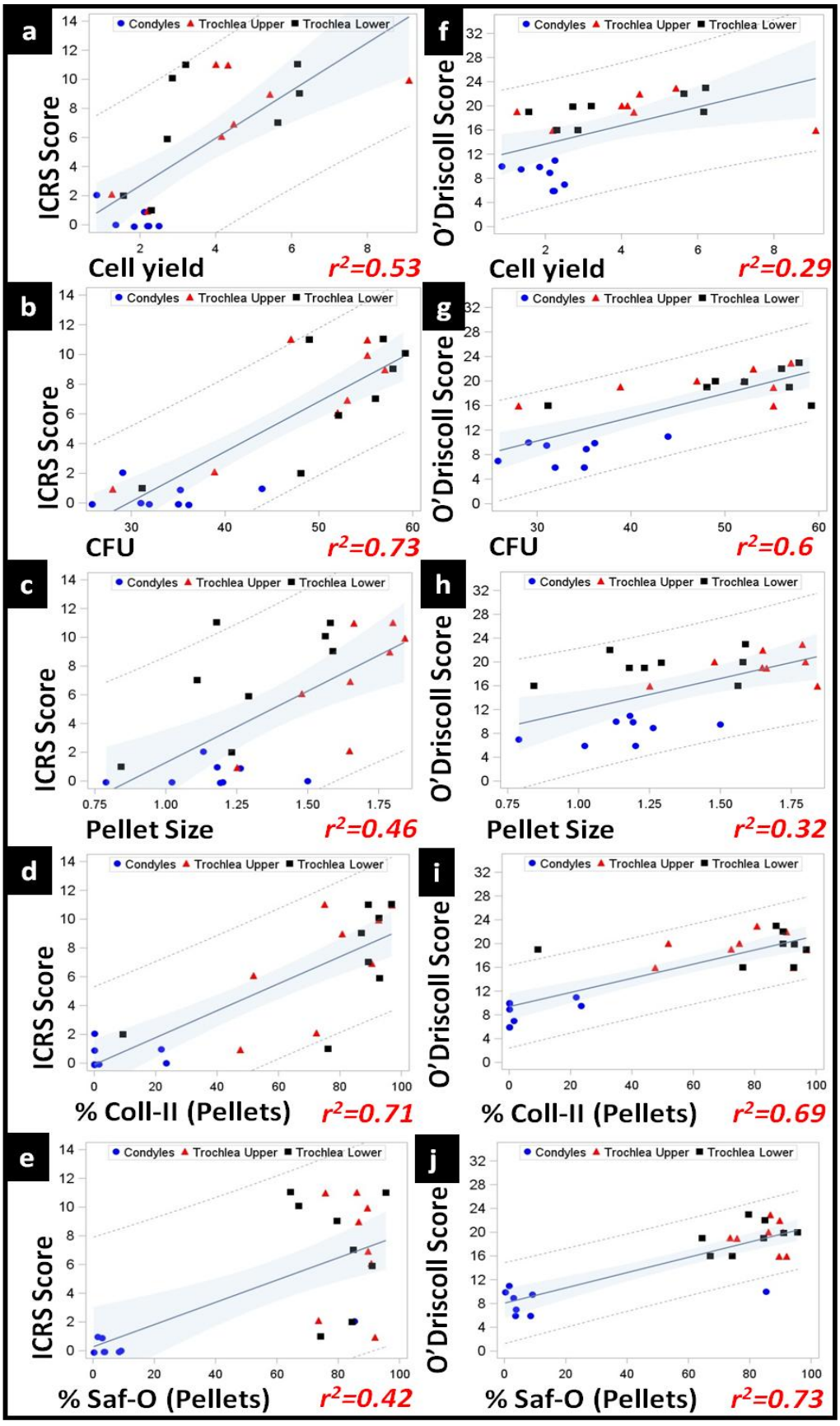
a
Condyles Trochlea Upper Trochlea Lower
ICRS Score
Cell yield
r²=0.53
f
Condyles Trochlea Upper Trochlea Lower
O'Driscoll Score
Cell yield
r²=0.29
b
Condyles Trochlea Upper Trochlea Lower
ICRS Score
CFU
r²=0.73
g
Condyles Trochlea Upper Trochlea Lower
O'Driscoll Score
CFU
r²=0.6
c
Condyles Trochlea Upper Trochlea Lower
ICRS Score
Pellet Size
r²=0.46
h
Condyles Trochlea Upper Trochlea Lower
O'Driscoll Score
Pellet Size
r²=0.32
d
Condyles Trochlea Upper Trochlea Lower
ICRS Score
% Coll-II (Pellets)
r²=0.71
i
Condyles Trochlea Upper Trochlea Lower
O'Driscoll Score
% Coll-II (Pellets)
r²=0.69
e
Condyles Trochlea Upper Trochlea Lower
ICRS Score
% Saf-O (Pellets)
r²=0.42
j
Condyles Trochlea Upper Trochlea Lower
O'Driscoll Score
% Saf-O (Pellets)
r²=0.73

Figure 5.6: Strong, positive correlation between in vitro biological properties of collagenase-derived BMSCs-Cell yield, CFU, pellet size, % Saf-O (pellets), % Coll-II (pellets)- and repair response measured by ICRS macroscopic scoring and O'Driscoll Score in trochlea vs. condyles. n=8. C.I-95%; $p<0.0001$-0.0068. (RT-repair tissues. Blue circles-Condyles, Red triangles-Trochlea Upper, Black squares-Trochlea Lower).

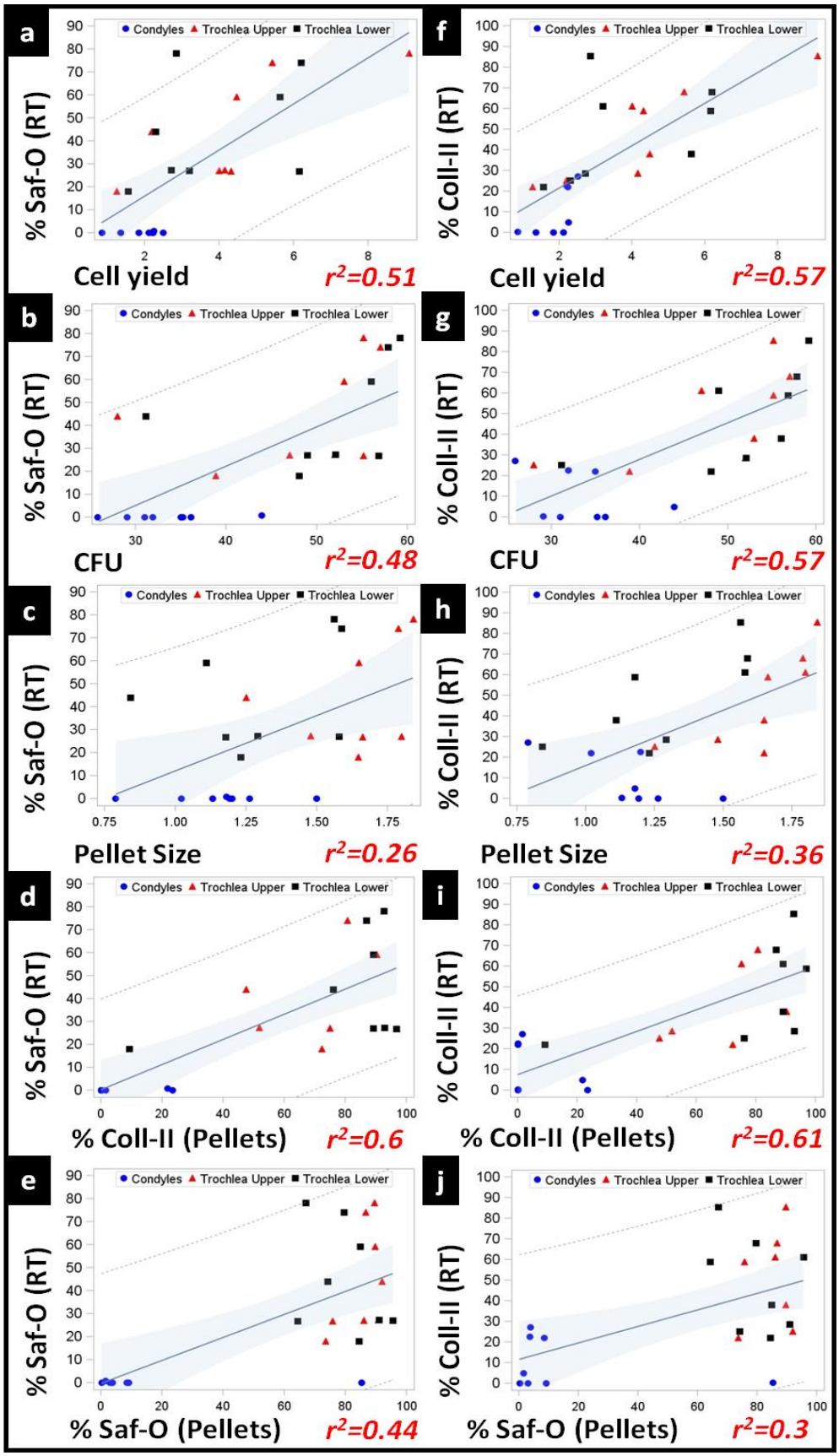
a
% Saf-O (RT)
Condyles Trochlea Upper Trochlea Lower
Cell yield
r²=0.51
f
% Coll-II (RT)
Condyles Trochlea Upper Trochlea Lower
Cell yield
r²=0.57
b
% Saf-O (RT)
Condyles Trochlea Upper Trochlea Lower
CFU
r²=0.48
g
% Coll-II (RT)
Condyles Trochlea Upper Trochlea Lower
CFU
r²=0.57
c
% Saf-O (RT)
Condyles Trochlea Upper Trochlea Lower
Pellet Size
r²=0.26
h
% Coll-II (RT)
Condyles Trochlea Upper Trochlea Lower
Pellet Size
r²=0.36
d
% Saf-O (RT)
Condyles Trochlea Upper Trochlea Lower
% Coll-II (Pellets)
r²=0.6
i
% Coll-II (RT)
Condyles Trochlea Upper Trochlea Lower
% Coll-II (Pellets)
r²=0.61
e
% Saf-O (RT)
Condyles Trochlea Upper Trochlea Lower
% Saf-O (Pellets)
r²=0.44
j
% Coll-II (RT)
Condyles Trochlea Upper Trochlea Lower
% Saf-O (Pellets)
r²=0.3

Figure 5.7: Strong, positive correlation between in vitro biological properties of collagenase-derived BMSCs-Cell yield, CFU, pellet size, % Saf-O (pellets), % Coll-II (pellets)- and repair response measured by % Saf-O (RT) and % Coll-II (RT) in trochlea vs. condyles. N=8. C.I-95%; $p<0.0001$-0.0068. (RT-repair tissues. Blue circles-Condyles, Red triangles-Trochlea Upper, black squares-Trochlea Lower).

In contrast, the *in vitro* biological properties of explant-derived BMSCs showed positive, significant ($p<0.5$) but weak correlations with *in vivo* characterization parameters (r^2 ranged from 0.1 to 0.3). As an exception, % Coll-II (pellets) of explant-derived BMSCs demonstrated strong, positive correlations with all the *in vivo* parameters (r^2 ranged from 0.38 to 0.56) [Fig 5.4S and 5.5S].

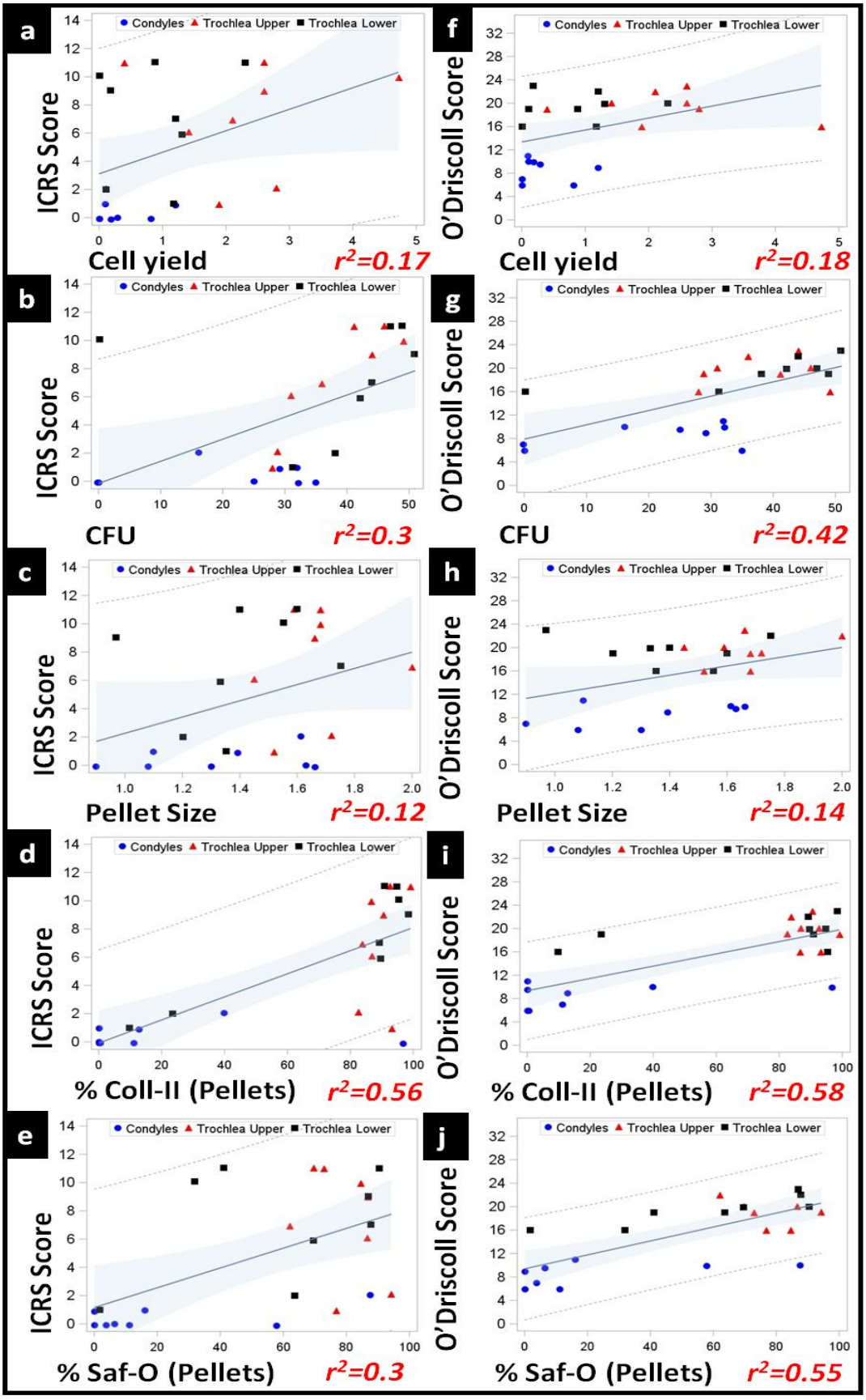
a
ICRS Score
Condyles Trochlea Upper Trochlea Lower
Cell yield
r²=0.17
f
O'Driscoll Score
Condyles Trochlea Upper Trochlea Lower
Cell yield
r²=0.18
b
ICRS Score
Condyles Trochlea Upper Trochlea Lower
CFU
r²=0.3
g
O'Driscoll Score
Condyles Trochlea Upper Trochlea Lower
CFU
r²=0.42
c
ICRS Score
Condyles Trochlea Upper Trochlea Lower
Pellet Size
r²=0.12
h
O'Driscoll Score
Condyles Trochlea Upper Trochlea Lower
Pellet Size
r²=0.14
d
ICRS Score
Condyles Trochlea Upper Trochlea Lower
% Coll-II (Pellets)
r²=0.56
i
O'Driscoll Score
Condyles Trochlea Upper Trochlea Lower
% Coll-II (Pellets)
r²=0.58
e
ICRS Score
Condyles Trochlea Upper Trochlea Lower
% Saf-O (Pellets)
r²=0.3
j
O'Driscoll Score
Condyles Trochlea Upper Trochlea Lower
% Saf-O (Pellets)
r²=0.55

Figure 5.4S: Weak, positive correlation between in vitro biological properties of Explant-derived BMSCs-Cell yield, CFU, pellet size, % Saf-O (pellets), % Coll-II (pellets)- and repair response measured by ICRS macroscopic scoring and O'Driscoll Score in trochlea vs. condyles. N=8. C.I-95%; $p<0.0001$-0.0068. (RT-repair tissues. Blue circles-Condyles, Red triangles-Trochlea Upper, Black squares-Trochlea Lower).

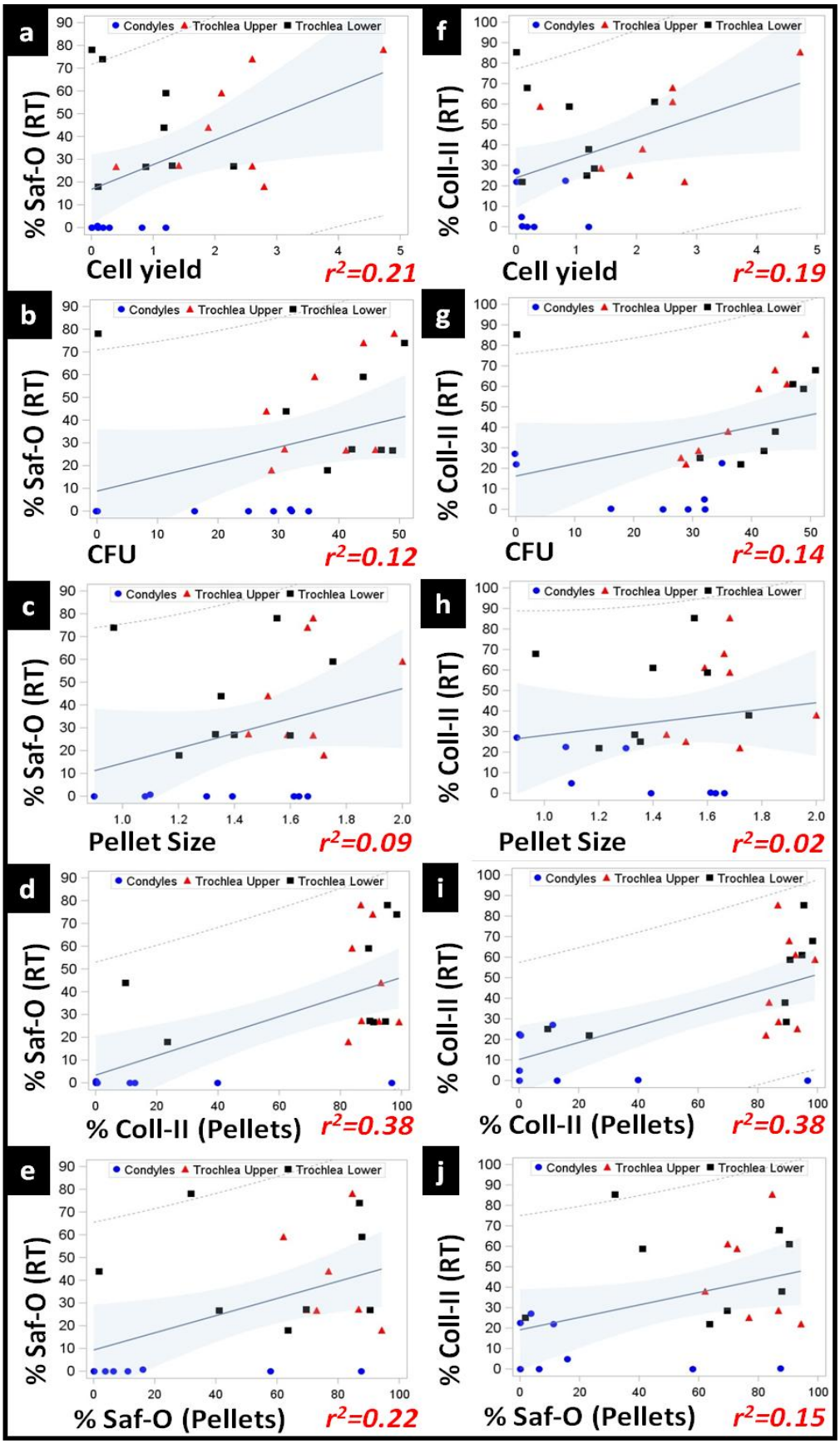
a
Condyles Trochlea Upper Trochlea Lower
% Saf-O (RT)
Cell yield
r²=0.21
f
Condyles Trochlea Upper Trochlea Lower
% Coll-II (RT)
Cell yield
r²=0.19
b
Condyles Trochlea Upper Trochlea Lower
% Saf-O (RT)
CFU
r²=0.12
g
Condyles Trochlea Upper Trochlea Lower
% Coll-II (RT)
CFU
r²=0.14
c
Condyles Trochlea Upper Trochlea Lower
% Saf-O (RT)
Pellet Size
r²=0.09
h
Condyles Trochlea Upper Trochlea Lower
% Coll-II (RT)
Pellet Size
r²=0.02
d
Condyles Trochlea Upper Trochlea Lower
% Saf-O (RT)
% Coll-II (Pellets)
r²=0.38
i
Condyles Trochlea Upper Trochlea Lower
% Coll-II (RT)
% Coll-II (Pellets)
r²=0.38
e
Condyles Trochlea Upper Trochlea Lower
% Saf-O (RT)
% Saf-O (Pellets)
r²=0.22
j
Condyles Trochlea Upper Trochlea Lower
% Coll-II (RT)
% Saf-O (Pellets)
r²=0.15

Figure 5.5S: Weak, positive correlation between in vitro biological properties of explant-derived BMSCs-Cell yield, CFU, pellet size, % Saf-O (pellets), % Coll-II (pellets)- and repair response measured by % Saf-O (RT) and % Coll-II (RT) in trochlea vs. condyles. N=8. C.I-95%; $p<0.0001$-0.0068. (RT-repair tissues. Blue circles-Condyles, Red triangles-Trochlea Upper, Black squares-Trochlea Lower).

GLM analysis confirmed that cell number, clonogenic and chondrogenic potential can serve as predictors of repair outcome. However, not all BMSC properties accounted for the variability in repair outcome to an equal extent [Table 5.1]. Macroscopic and O'Driscoll scores were better predicted by clonogenic and chondrogenic potential of pellets (% Saf-O and % Coll-II). On the other hand, matrix content of repair tissues- % Saf-O and % Coll-II- was more predicted by the initial cell population density (P0 cell yield) and chondrogenic potential of pellets (also defined by %Saf-O and % Coll-II). Taken together, cell yield, CFU and % Coll-II provided the maximum explanation for variability in different *in vivo* repair parameters. Consistent with these GLM analyses, these variables also presented the highest r^2 values in the independent pair-wise Pearson correlation analysis [Table 5.1, table 5.2S and Figs 5.6, 5.7].

Table 5.1: GLM analysis of dependent variables to determine the variables with maximum influence on repair outcome. First column denotes the variables with least influence on the repair outcome, removed sequentially in the order of significance. The resulting r^2 value after removal of each variable is outlined in column 3. Significant variables providing the maximum explanation for variability in repair outcome parameters are summarized in column 4 with their respective p values in column 5.

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Parameters excluded sequentially (in order of significance, p)	p value of less significant variables	Resulting collective r^2	Significant variables	p value of significant variables
ICRS Score				
None	--	0.85		
Saf-O (pellets)	0.52	0.84	CFU	<0.0001
Cell yield	0.26	0.83	Coll-II (Pellets)	0.005
Pellet Size	0.14	**0.81**		
O'Driscoll Score				
None	--	0.87		
Pellet Size	0.9	0.87	CFU	0.0002
Cell yield	0.32	0.86	Saf-O (pellets)	<0.0001
Coll-II (pellets)	0.19	**0.85**		
%Saf-O (repair tissue)				
None	--	0.7		
Pellet Size	0.9	0.7	Cell yield	0.04
CFU	0.82	0.7	Coll-II (Pellets)	0.0033
%Saf-O (pellets)	0.16	**0.67**		
% Coll-II (repair tissue)				
None	--	0.73		
Saf-O (pellets)	0.97	0.73	Cell yield	0.0096
CFU	0.57	0.72	Coll-II (Pellets)	0.005
Pellet Size	0.38	**0.71**		

Table 5.2S: Individual pair-wise correlation coefficients between independent and dependent variables with corresponding p values for condyles, trochlea upper and trochlea lower. Values highlighted in green denote significant correlations between *in vivo* parameters - ICRS score, O'Driscoll score, %Saf-O RT (repair tissue) and %Coll-II RT (repair tissue) – and *in vitro* parameters including cell yield, CFU-f, pellet size, Saf-O pellets and Coll-II pellets. (Saf-O and Coll-II denote % Saf-O and % Coll-II respectively).

	ICRS Score		ODriscoll		Saf-O RT		Coll-II RT	
Condyles								
Cell Yield	-0.55		-0.49		0.15		0.65	
CFU-f	0.1		0.44		0.75	0.03	-0.33	
Pellet Size	0.06		0.45		0.03		-0.7	0.05
Saf-O pellets	0.77	0.02	0.25		-0.07		-0.28	
Coll-II pellets	-0.02		0.5		0.6		-0.3	
Trochlea upper								
Cell Yield	0.68		-0.09		0.74	0.03	0.86	0.0059
CFU-f	0.81	0.01	0.52		0.39		0.65	
Pellet Size	0.76	0.02	0.35		0.33		0.77	0.02
Saf-O pellets	-0.03		-0.09		0.53		0.08	
Coll-II pellets	0.7	0.05	0.22		0.36		0.68	
Trochlea Lower								
Cell Yield	0.59		0.63		0.37		0.41	
CFU-f	0.75	0.03	0.48		0.36		0.63	
Pellet Size	0.7	0.05	0.31		0.31		0.75	0.03
Saf-O pellets	-0.12		0.49		-0.37		-0.39	
Coll-II pellets	0.65		0.1		0.41		0.53	

5.4 Discussion

For the first time, this study provides tangible evidence of a direct correlation between marrow stimulated repair outcome in condyle and trochlea and inherent biological properties of BMSCs present in these two locations. Variability in cell number, clonogenic and chondrogenic potential of trochlear BMSCs showed strong correlations with quality and quantity of repair tissue in a location- and donor-dependent manner. Cell number, clonogenic and matrix production potential of BMSCs were strong predictors of macroscopic and histological repair outcomes. We found sufficient evidence to substantiate our hypothesis that trochlear cartilage repair outcome is superior to condyles and correlates with *in vitro* biological properties of the BMSCs at the two sites in the contralateral knee.

Bone marrow stimulated repair is mediated by recruitment of MSCs into access channels and cartilage defect followed by a complex multistep cascade of events including proliferation, cell-cell interaction and differentiation finally leading to formation of a repair tissue with varying amounts of fibrous and hyaline cartilage [17, 18]. It is logical to speculate that properties of MSCs will have a profound impact on the repair outcome. We believe that the differences in the initial progenitor cell population have a strong bearing on the repair response. As shown by Mizuta *et al* in a rabbit full-thickness defect model, explicit chondrogenesis was preceded by an active phase of expansion in chondroprogenitor cell population [19]. Moreover, a high number of cells in the initial stages of chondrogenesis is a prerequisite since close interaction of closely packed cells during condensation phase is critical for initiation of chondrogenic differentiation of MSCs [20]. Fennema *et al* had reported that the differences in the original number of proliferative cells isolated were mirrored in the growth rate of corresponding BMSC cultures [21]. In the current study, lower cell yield indicated a depleted progenitor cell population in condyles having an impact on the onset and progress of downstream repair process. Low number accompanied with reduced clonogenic potential as indicated by CFU-f assay might attribute to diminished chondrogenic potential of condyles. It has also been shown that increasing population doublings escalate heterogeneity, reduces multipotency and repair potential [22]. Inferior chondrogenic potential of condylar pellets might also be attributed to poor response of BMSCs to chondrogenic stimulation due to molecular factors or being in different stages of lineage progression.

In vitro cultures of BMSCs provide limited insight into the complex and dynamic joint microenvironment during repair, however studies have shown that cellular behaviour of MSC cultures provide close estimation of their *in vivo* potency and allude to heterogeneity in the repair outcome [23]. Post *et al* characterized two murine MSC populations *in vitro* and found a pre-existing propensity towards adipogenic or osteogenic differentiation which was consistent with *in vivo* behavior [24]. Moreover, MSC cultures have been shown to be heterogeneous with respect to morphology [25] and consist of rapidly dividing small cells and slower, larger cells reflecting correlation in proliferation rates and morphological heterogeneity. This morphological heterogeneity was later correlated to functional heterogeneity *in vitro* as well as *in vivo* [26]. Lee *et al* had demonstrated association between cell diameter, mechanical stiffness and fluctuations of nuclear membrane with clonogenic and proliferation potential in addition to potential for *in vitro* differentiation potency and repair *in vivo* [27]. Further, on comparison of stem cells from variety of niches, Woo *et al* revealed marked differences in their proliferation and differentiation propensity despite similar cell surface marker profiles [28]. Authors attributed these observations to cellular environment and genetic factors including epigenetic factors. Although the mechanism is not clear, epigenetic factors regulating stem cell differentiation alter fate of cells from different locations underlying the influence of location on their inherent nature [29]. Stem cells isolated from different locations have also shown differences in their propensities towards a particular lineage-such as preference for adipogenic and osteogenic differentiation in the case of adipose derived stem cells and BMSC respectively [28]. Furthermore, studies have shown that proliferative and functional profile of MSCs are altered in OA [30, 31]. Taking together these observations, we can conclude location-dependent variation in cartilage repair response is most likely due to inherent differences in the biological properties of subchondral BMSCs including their number and differentiation potential.

In another observation, repair outcome in poor trochlear responders was associated with inferior number, clonogenic and differentiation potential of BMSCs isolated from trochlea of these donors. Earlier studies have reported cultures obtained from different donors to be heterogenous with respect to number and differentiation [32, 33]. Interestingly, our data suggests that inferior

repair in poor trochlear responders may be a result of a depleted progenitor cell population further aggravated by a reduced clonogenic potential of BMSCs isolated from metaphyseal region of trochlea in these donors. These probably had a direct bearing on the chondrogenic potential indicated by depleted GAG expression in trochlea lower pellets. Smaller pellet size further suggests that inferior chondrogenic differentiation potential of metaphyseal BMSCs might be responsible for poor chondrogenic differentiation in poor trochlea responders.The poor potential of these donors seems to be at least partially intrinsic since the cells also had reduced expression of cell surface markers. These observations provide further evidence of correlation of cartilage repair response and inherent properties of local population of BMSCs in the region.

Although moderate to strong positive and significant Pearson correlation coefficients were observed between all the biological properties analysed and corresponding repair outcome parameters, GLM analysis revealed that certain correlations were more strongly defined by trochlea data. Taken together, trochlea upper provided maximum explanation for the variability observed *in vivo* [Table 5.2S]. Weak correlations observed with explant-derived BMSCs might suggest a limited contribution of these cells in cartilage repair, likely restricted by their ability to migrate out of bone. Due to low initial yield, these cells might have undergone more population doublings in culture leading to loss in stemness and subsequent chondrogenic potential.

It is important to bear in mind that repair of a cartilage defect does not occur in isolation and is affected by surrounding milieu which provides important signals by means of cellular interactions, growth and differentiation factors in addition to other mechanical and chemical stimuli. These factors impact the signaling pathways which control transcription of specific genes contributing to onset and progress of repair. For instance, Wnt proteins, TGF-β superfamily, BMPs, FGF, IGF are some of the several morphogenetic factors [34] which influence BMSCs possibly controlling the eventual repair outcome. Moreover, *in vitro* expansion and differentiation of BMSCs has been shown to be influenced by hydrostatic, compression and shear forces [35-39] in addition to mechanical loading and oxygen tension [40]. The downstream differentiation commitment of BMSCs may also be influenced by spatial dimensionality modifying the interaction of BMSCs in trochlea and condyle and their corresponding

microenvironment [41]. In addition, the mechanical environment plays a role in regulation of TIMPs and MMPs which have been implicated in the differentiation of BMSCs [42, 43]. Besides, ECM components regulate adhesion and survival of BMSCs and thus have an impact on the downstream commitment of BMSCs [44]. Taken together, there is a strong possibility that *in vivo* behaviour of BMSCs might be significantly influenced by location as a result of striking structural and anatomical differences between condyle and trochlea. This may alter the mechanical and chemical cues which have an important role in regulating the cellular behaviour of BMSCs in trochlea and condyle.

5.5 Conclusion

Through this study we found substantial evidence to suggest that clear differences exist in biological properties of BMSCs present in condyles and trochlea and that these differences are most likely the cause of variation in the repair outcome observed in these two locations. Although *in vitro* cultures of BMSCs can be coaxed into multiple phenotypes, they might not be an absolute reflection of their inherent potential. This might be further complicated by differences in the receptor profile of cells *in vivo* which might alter the response mediated by signaling pathways impossible to replicate *in vitro*. However, since precise methods for *in vivo* identification of BMSC and their behavioural patterns still elude us, it becomes imperative to carry out functional phenotype analysis both *in vitro* and *in vivo* in order to draw parallels between *in vitro* behaviour of BMSCs and corresponding repair outcome in an animal model. In the future, we plan to carry out detailed molecular profiling of BMSCs in order to identify the genetic mechanism of the location- and donor-dependent differences. Here, we were able to discern important mechanistic insight into underlying cause of location- and donor-related variation observed with BMS repair outcomes. We speculate our results will advance our understanding of donor- and site-specific variations in BMS repair response eventually helping in maximizing the efficiency of procedure in a more patient relevant basis.

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CHAPTER 6 ARTICLE 3: FREEZE-DRIED CHITOSAN/PRP IMPLANTS IMPROVE MARROW STIMULATED CARTILAGE REPAIR IN A CHRONIC DEFECT RABBIT MODEL

The previous studies enabled us to improve our understanding of characteristics of MSCs present in condyle and trochlea and present a likely mechanism of donor-, age- and location-dependent variability in marrow stimulated repair outcome. BMS has been known to be highly unpredicatable, and generate a fibrocartilagenous repair tissue with poor durability. Moreover, preclinical studies for cartilage repair strategies are generally performed in an acute model which is not an accurate representation of clinical situation leading to unsuccessful clinical translation of promising strategies. We recognised the need to develop a model with chronic, degenerative lesions for better clinical representation. Augmentation strategies have been shown to exert a positive impact in enhancing the outcome of BMS procedure. Platelet-rich-plasma (PRP) with its 4-fold concentration of growth factors and cytokines promotes MSC proliferation and differentiation. However, burst release of factors and poor stability likely lead to inferior outcomes. Chitosan has been shown to increase the stability of blood clot formed in the defect and therefore improves the repair outcome. Combined with PRP, Chitosan is likely to form a voluminous, stable clot capable of sustained release of bioactive factors and superior retention of growth factors and MSCs in the defect. In this article, a chronic, bilateral model was developed using skeletally mature rabbits. BMS augmented with recalcified PRP and chitosan/PRP implants were compared. In addition to macroscopic assessment, two month repair was analyzed histologically to compare matrix composition. MicroCT analysis enabled understanding of subchondral bone regeneration and remodeling induced by two treatments. The conclusions from this study are expected to be of significant clinical relevance and eventually enable successful clinical translation of a promising approach. *Submitted-Biomaterials*.

Freeze-dried chitosan/PRP implants improve marrow stimulated cartilage repair in a chronic defect rabbit model

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Running title: Chitosan/PRP for cartilage repair in a chronic model

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Abstract

Purpose

Bone Marrow Stimulation (BMS) improves knee joint function but elicits incomplete repair. Liquid chitosan (CS)-glycerol phosphate/blood clots have been shown to improve BMS-based cartilage repair. Platelet-rich-plasma (PRP) – a rich source of growth factors and cytokines - improves recruitment and chondrogenic potential of subchondral mesenchymal stem cells. We hypothesized that repair response in a rabbit chronic defect model will improve when freeze-dried chitosan/PRP is used to augment BMS.

Methods

Bilateral trochlear defects created in New Zealand White rabbits were allowed to progress to a chronic stage over 4 weeks. Chronic defects were debrided and treated by BMS in second surgery, then augmented with PRP (BMS+PRP) or chitosan/PRP implants (BMS+CS/PRP). The quality of 8 week repair tissue was assessed by macroscopic, histological and micro-CT analysis.

Results

ICRS macroscopic scores indicated fibrocartilaginous or fibrous repair in control defects that were improved in the BMS+CS/PRP group. An overall improvement in repair in BMS+CS/PRP group was further confirmed by higher O'Driscoll scores, %Saf-O and %Coll-II values. Micro-CT analysis of subchondral bone indicated on-going remodeling with repair still underway.

Conclusion

Quality and quantity of cartilage repair was improved when chitosan/PRP implants were used to augment BMS in a chronic defect model.

6.1 Introduction

Bone marrow stimulation (BMS) is a purely surgical process which initiates cartilage repair by fracturing or drilling into subchondral bone. BMS procedures initiate the formation of a blood clot around fractured bone followed by migration of subchondral progenitor cells which differentiate into a chondrogenic phenotype to form a repair tissue with variable amounts of hyaline and fibrous cartilage [1]. The repair tissue is typically characterized by low quality and compromised durability, and larger lesions in older patients are even more challenging to treat.

One possible reason for the poor performance of BMS is that the blood clot rapidly shrinks to a fraction of its initial size due to platelet-mediated clot retraction, resulting in lack of defect filling and possible detachment from the tissue surface. One way to prevent clot retraction is to add chitosan (CS), a polymer of glucosamine and N-acetyl glucosamine units, to the blood [2]. Liquid CS-glycerol phosphate (GP)/blood implants can be applied over BMS-treated cartilage defects where they coagulate *in situ* and inhibit platelet-mediated clot retraction leading to the formation of a voluminous, adherent and physically stable clot with access to underlying marrow [3]. When used in conjunction with BMS procedures, CS-GP/blood implants promote cell recruitment, transient vascularization and subchondral bone remodeling leading to integrated repair and increased hyaline character of the repair tissue [2-5]. These implants were tested clinically [6, 7] and have now been approved in several countries to treat cartilage lesions (BST-CarGel®, Smith and Nephew, USA). One drawback of this technology is that liquid CS solutions have limited stability during storage due to acid hydrolysis of CS and loss of viscosity [8]. A freeze–dried form of CS would not only overcome this limitation by increasing stability and shelf life but also permit easier sterilization.

A microenvironment stimulating chondrogenic differentiation of marrow-derived mesenchymal stem cells (BMSCs) may be achieved by addition of platelet-rich-plasma (PRP) to the defect milieu. PRP is prepared by sequential centrifugation of whole blood and is a rich source of growth factors and cytokines such as PDGF, VEGF, IGF, EGF etc which play an important role in inflammatory and wound repair phenomena [9-11]. Earlier studies have shown that PRP can induce a significant improvement in BMSC recruitment, angiogenesis [12, 13], expression of

cartilage matrix [14], proliferation and viability of chondrocytes and BMSCs [15-16] as well as stimulate migration and chondrogenic potential of subchondral BMSCs [17]. Using an ovine chronic defect model, Milano *et al* showed that when used as an adjunct to BMS, PRP enhanced the repair response versus BMS alone [18]. Although the application of PRP improved the macroscopic and mechanical outcome, the hyaline nature of the repair tissue was still lacking. The efficacy of PRP in improving cartilage repair has been questioned due to multiple studies reporting less positive results in animal models [19-22]. We believe that inferior outcome with PRP could arise from the poor stability of PRP clots *in vivo*, which is even more pronounced than blood clots [23]. Combination of PRP with CS may help in overcoming this limitation thereby increasing residency and bioactivity of PRP.

Progression to advanced stages of osteoarthritis (OA) may be prevented by early diagnosis and treatment. However, in cartilage lesions that are asymptomatic for longer times [24,25], BMS may have more severe limitations and therefore be less effective in treating older, chronic and extensive lesions. Chronic defects may alter joint homeostasis resulting in less favourable clinical outcomes [26-28]. To better represent this clinical situation, we developed a pre-clinical model to simulate degenerated chronic defects and examine the potential of BMS combined with CS/PRP in improvement of cartilage regeneration in chronic defects that are more challenging than acute lesions. It is already known that osteochondral defects smaller than 3 mm in young rabbits possess the potential for spontaneous regeneration [29] and several studies have reported a period of approximately 1 month to be adequate for development of chronic defect model in small animals [30,31]. Accordingly, we used skeletally mature rabbits with a defect size of 4 × 4 mm developed to chronicity over a period of four weeks. With these aspects in mind we carried out this study to test the hypothesis that augmentation of BMS with freeze-dried CS/PRP implants would improve repair response in a rabbit chronic defect model compared to BMS augmented with recalcified PRP.

6.2 Materials and Methods

6.2.1 Preparation of freeze-dried chitosan formulation and PRP isolation

Freeze-dried CS cakes consisted of 0.56% (w/vol) CS with 1% (w/vol) trehalose and 42.2 mM calcium chloride. To prepare, 0.056 g CS (Number average molar mass M_n 36.6 kDA and 80.2% DDA, produced in-house and characterized with NMR spectroscopy [32] and size-exclusion chromatography/multi-angle laser light scattering [33]) was mixed with 7.69 g water and 156 µl of 1 N HCl. Following overnight mixing, 1.56 ml of 3% (w/w) $CaCl_2$ and 666 µl of 15% (w/v) trehalose solutions were added and the final solution was sterilized by filtration. Finally, 300 µl aliquots were prepared in 2 ml sterile glass vials and freeze-dried using the following conditions: 1) Ramped freezing to -40^{o}C in 1 hour then isothermal 2 hours at -40^{o}C, 2) -40^{o}C for 48 hours at 100 millitorrs and 3) Ramped heating to 30^{o}C in 12 hours then isothermal 6 hours at 30^{o}C, at 100 millitorrs.

Autologous PRP was generated by sequential centrifugation of citrate-anticoagulated whole blood. Approximately 9 ml of autologous blood was extracted from rabbit and mixed with 1 ml of 3.8% (w/vol) sodium citrate before further processing. The whole blood was centrifuged at 160g for 10 minutes. Following collection of the supernatant in addition to approximately the first 1-2 mm of erythrocytes, a second centrifugation was carried out at 400g for 10 minutes. Bottom 1.5 ml fraction containing PRP was isolated. Complete blood counts revealed that, on average, the ratio of platelets, leukocytes and erythrocytes in isolated PRP versus whole blood was 3X, 1X and 0.1X, respectively, which makes this a leukocyte-rich PRP.

6.2.2 Experimental design and rabbit surgical model for cartilage repair in chronic lesions

Canadian Council on Animal Care guidelines were observed and research protocol was approved by an institutional ethics committee for animal research. Using skeletally mature female New Zealand White rabbits, the chronic defect model was first validated in a pilot study utilizing a small number of animals (n=3) and short duration (4 weeks development to chronicity followed

by 3 weeks repair). The objective was to assess the feasibility of development of the model as well as compare fresh chronic defects (n=2 knees) to chronic defects after 4 weeks development (n=2 knees) and repair responses induced in chronic defects by BMS alone (n=1 knee) and BMS augmented with 1% (w/v) CS/PRP implants (n=1 knee). PRP controls were not included in this pilot study since the aim was to validate the chronic model rather than study the effect of treatments.

A larger group (n=8) of skeletally mature (8-9 months old) female New Zealand White rabbits were then used in a bilateral model that limits the influence of inter-animal variation via a contralateral control. Two surgeries were performed to assess the development and repair of chronic cartilage defects. Following induction with xylazine-ketamine, animals were maintained under general anaesthesia using isofluorane-oxygen. Bilateral, parapatellar arthrotomies were performed to expose the synovial joint. Full-thickness chondral lesions measuring 4 × 4 mm were created in the trochlear central groove by scraping with 1.5 and 2.75 mm flat surgical blades taking care not to remove calcified cartilage. Knees were closed in sutured layers. A second surgery was performed four weeks later and the original lesion identified. Calcified cartilage (CC) and repair tissues (when present) were completely debrided using a flat blade to expose the underlying subchondral bone, without damaging the subchondral bone plate. Using a high speed microdrill, four subchondral perforations measuring 0.9 mm diameter and 6 mm deep were made on each trochlear defect in both knees, similar to what was done previously by our group in acute models [34,35]. Constant cooling irrigation was applied for removal of loose bone debris and prevention of heat necrosis [36]. The defect on one knee received treatment with one drop of CS/PRP mixture. Immediately before implantation, the CS cake (300 µL) was solubilized with 300 µL autologous PRP. One drop of CS/PRP was applied to the drilled trochlear defect using a 1 ml syringe and 18 gauge needle. The contralateral defect was treated with one drop of PRP recalcified with 42.2 mM calcium chloride. In both cases, knees were closed five minutes post application. The treatments were alternated between right and left knees [Fig. 6.1]. The patella was repositioned and knee was closed in sutured layers. No perioperative antibiotics were administered after either surgery but animals received extended analgesia with a fentanyl transdermal patch. Knees were allowed unrestricted motion and constantly monitored for infections and other complications following both surgeries. Animals were sacrificed eight weeks

later and knees were harvested for comparison of marrow stimulated repair response in presence of CS/PRP (BMS+CS/PRP group, n=8 knees) and recalcified PRP (BMS+PRP group, n=8 knees).

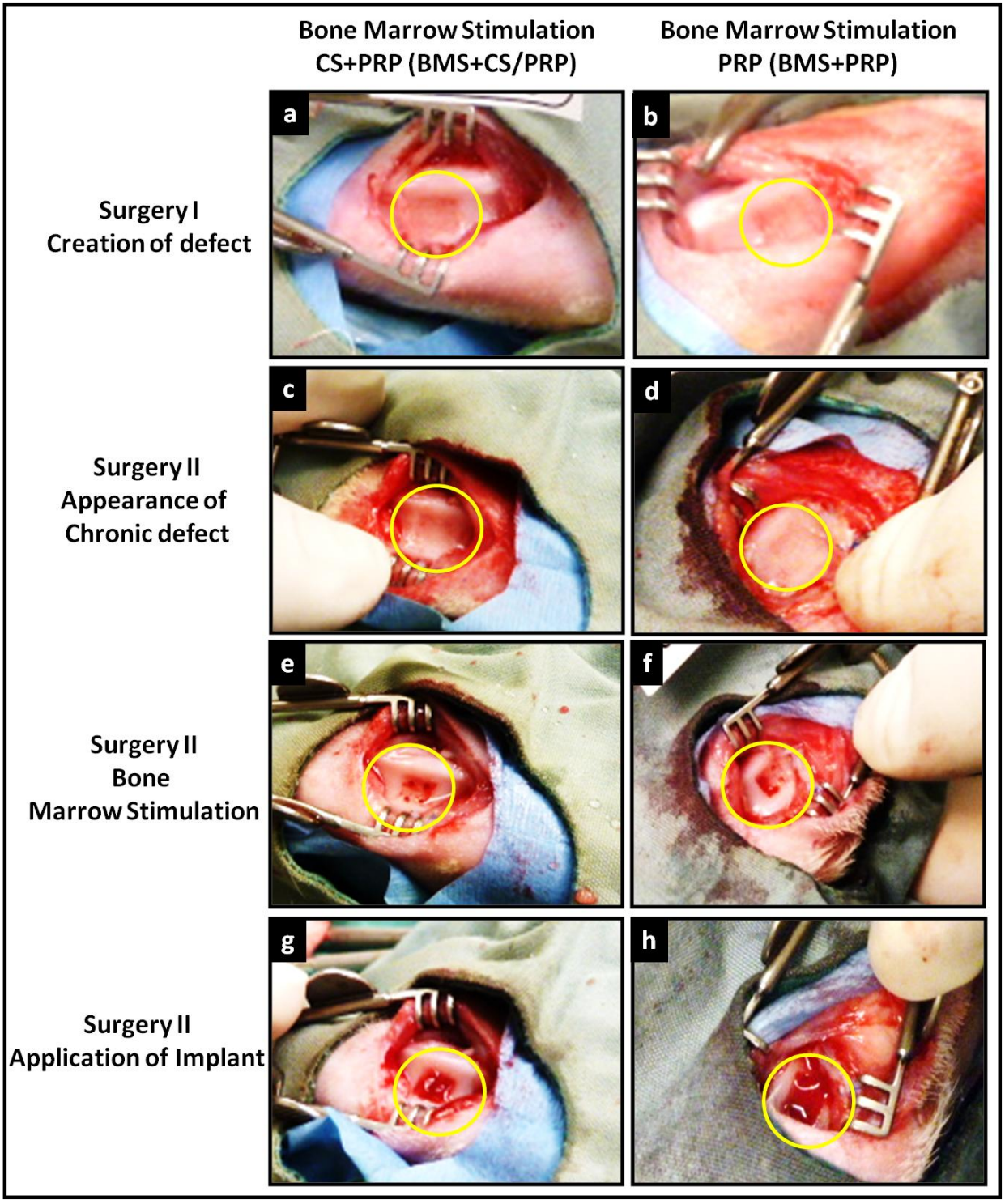


Figure 6.1: Procedure of surgical manipulation to create and treat chronic defects using BMS+CS/PRP implants (left panels) or BMS+PRP (right panels). a,b: Creation of 4 × 4 mm defects by debriding all non-calcified cartilage from trochlea; c,d: Appearance of chronic defects four weeks after creation at the time of second surgery; e,f: Treatment of defects by debriding spontaneous repair tissue (when present) and calcified cartilage and drilling 4 holes measuring 0.9 mm in width and 6 mm deep; g: Application of CS/PRP implant at defect site; h: Application of recalcified PRP at defect site.

6.2.3 Characterization of repair

Animals were sacrificed by overdose of pentobarbital and femoral ends were fixed in 4% paraformaldehyde /1%glutaraldehyde /0.1M Sodium cacodylate (pH 7.3). Low magnification images of fixed repair tissues were obtained with a dissection microscope equipped with digital camera to determine their gross structure and appearance using Northern Eclipse software (Empix Imaging). Images of repair tissues were scored by two independent, blinded readers using the ICRS macroscopic scoring system (ranging from 0, for severely abnormal to 12, for normal) [37]. Scores from two readers were averaged and used as an indicator of gross pathology of repair tissues.

Samples were decalcified in EDTA with trace paraformaldehye and embedded in OCT and transverse sections were obtained from three levels: from the middle of the proximal and distal holes and from between holes. Sections were stained with Safranin O/Fast Green and scanned using a Nanozoomer RS system (Hamamatsu, Japan). Repair tissue was defined as all non-mineralized tissue above the subchondral bone plate. Digital Saf-O stained sections were scored by two independent, blinded observers using a previously published O'Driscoll histological scoring method (ranging from 0, for worst tissue quality to 27, for best tissue quality) modified to assess subchondral bone health by an additional 3 point value [5]. Each Saf-O stained section was assessed for 10 criteria (Fig. 6.6c) to evaluate quality of repair tissue in addition to health of adjacent cartilage and subchondral bone repair. The scores obtained from three sections were

averaged for both readers and used for assessment of quality of fill in defects (ICC of 0.86 for total O'Driscoll score for both readers).

Sections collected from each defect were also used to determine %Saf-O and %Coll-II in the chondral repair tissue using a previously described method [38]. Briefly, soft repair tissues were cropped by identifying projected articular surface and tidemark with the help of flanking articular cartilage and accounting for the curvature of the trochlear groove. Only the soft repair tissue above the tidemark was used to determine hue-saturation-value threshold limits for Saf-O and Coll-II. %Saf-O and %Coll-II positive region of repair tissues were measured using in-house Matlab routine software.

6.2.4 MicroCT analysis of subchondral bone repair

Micro CT scanning of fixed femur ends was done to characterize subchondral bone repair and remodelling (Skyscan x-ray microtomography 1172, Kontich, Belgium). Femurs were scanned with an aluminium filter at 14.1 µM pixel size resolution with an X-ray source voltage of 56 kV, 1180 BMSec exposure, 0.45 rotation steps and 3 averaging frames. Trochlear micro CT image stacks were first reconstructed with NRecon software 1.6.1.5 using the following parameters: Smoothing of 2, Ring artifact reduction of 10, Beam hardening correction of 40%. Datasets were repositioned with DataViewer software 1.4.3 and region of interest were applied followed by 3D micro CT analysis. The regions of interest were of the rectangle adapted surface type [39] and measured 3 mm X 3 mm X 2 mm [Fig. 6.1S]. Bone morphometric parameters were calculated including bone surface density (BS/TV), bone surface (BS), bone volume (BV), porosity, connectivity density and number and thickness of trabeculae, by using the global thresholding procedure in CTAn software (version 1.9.3.0, Skyscan, Kontich, Belgium).

6.2.5 Statistical Analysis

Statistical analyses were performed using SAS Enterprise Guide 7.1 and SAS 9.4. Since several sections were collected from both legs of each rabbit, a mixed model was used to account for the influence of donor. Fixed effects were treatments (BMS+PRP, n=8 knees and BMS+CS/PRP, n=8 knees), while donor was a random effect. Data in figures are presented as mean (diamond); median (line); Box: 25^{th} and 75^{th} percentile; Whiskers: Box to the most extreme point within 1.5 interquartile range. $p<0.05$ was considered statistically significant.

6.3 Results

6.3.1 Freeze dried chitosan/PRP implants induced inflammatory and wound bloom repair responses in chronic cartilage defects

Although debridement appeared to have preserved the calcified cartilage intact due to absence of any punctuate bleeding at the time of initial surgery [Fig. 6.1a,b], the histological examination of Saf-O stained transverse sections of fresh defects showed that calcified cartilage had been partly debrided [Fig. 6.2e,i]. Chronic defects were noticeably distinct from the surrounding healthy tissue after 4 weeks [Fig. 6.1c,d], and residual calcified cartilage along with spontaneous repair response arising from the bone was occasionally observed [Fig. 6.2f,j]. Complete debridement down to subchondral bone was performed prior to BMS [Fig. 6.1e,f] and application of either CS/PRP [Fig. 6.1g] or recalcified PRP [Fig. 6.1h].

Defects treated with BMS+CS/PRP showed incomplete repair at 3 weeks [Fig. 6.2c] and histological examination of Saf-O stained transverse sections taken through holes revealed depressed repair tissues and enlarged remodeling drill holes [Fig. 6.2g,k], reminiscent of a wound bloom repair response [23]. Drill holes were devoid of subchondral cartilage and mostly filled with a polymorphonuclear cell-rich granulation tissue, where neutrophils colocalized with CS [Fig. 6.2g,k]. Contralateral defects treated with BMS alone also demonstrated incomplete repair at 3 weeks [Fig. 6.2d] and histological examination showed fibrocartilaginous and endochondral ossification repair responses, associated with chondrocyte hypertrophy and vascular invasion [Fig. 6.2h,l].

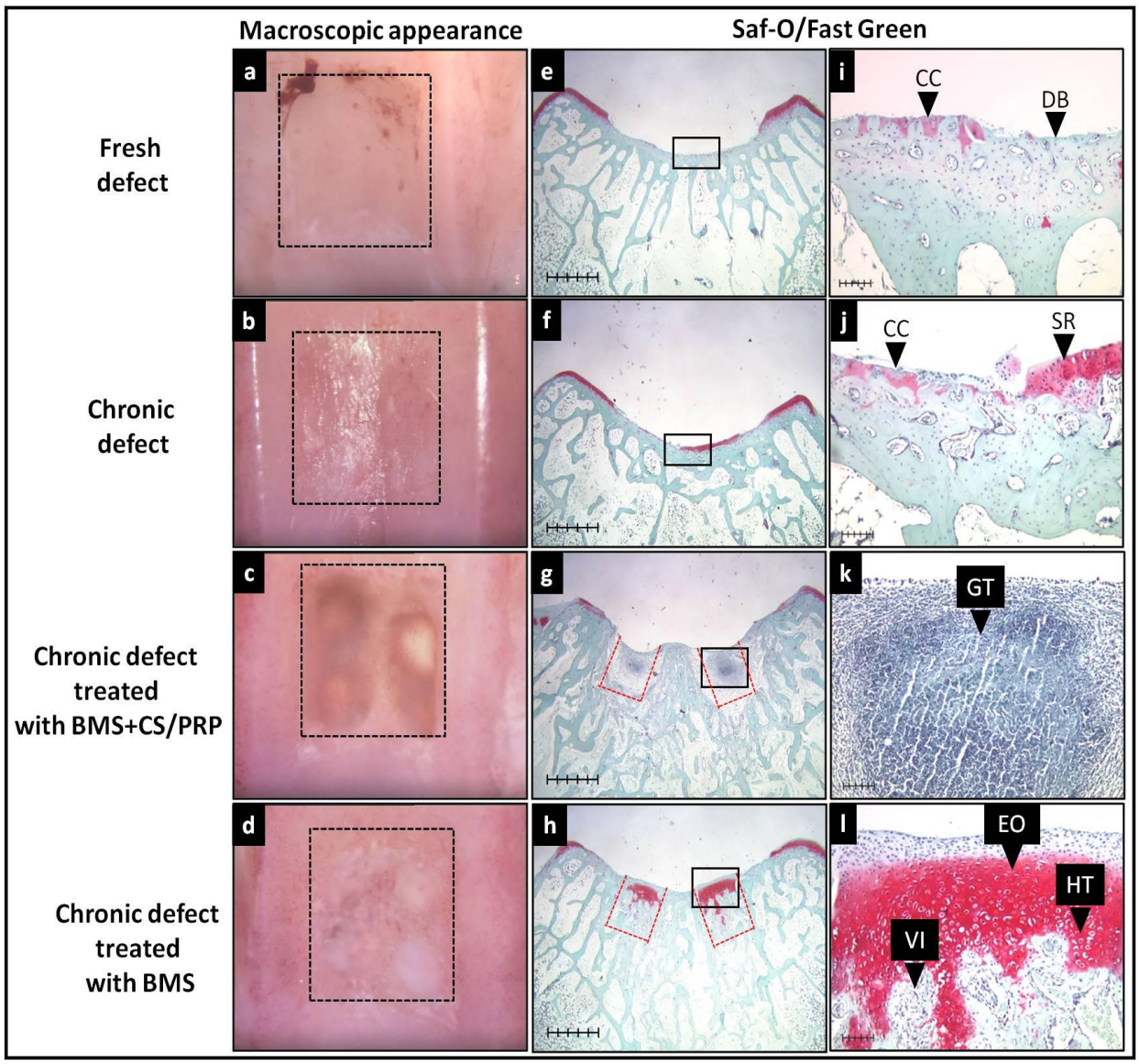


Figure 6.2: Macroscopic and histopathological assessment of fresh defect (a,e,i), chronic defect after 4 weeks development (b,f,j), chronic defect treated with BMS+CS/PRP implant (c,g,k) and chronic defect treated with BMS alone (d,h,l). (e,i): Debridement was not homogenous and varying levels of calcified cartilage (CC) and debrided bone (DB) were seen in freshly debrided defects. (f,j): After 4 weeks, chronic defects showed evidence of partial spontaneous repair (SR) in some areas along with tufts of calcified cartilage (CC). (g,k): Granulation tissue formation (GT) and enlarged drill holes were seen in presence of CS/PRP implants. (h,l): Fibrocartilagenous repair and endochondral ossification (EO) process were seen in presence of

BMS alone, associated with chondrocyte hypertrophy (HT) and vascular invasion (VI). Red dotted lines in g & h represent original drill holes- hole enlargement and wound bloom effect is apparent in defect treated with BMS+CS/PRP (g). Scale bar (e-h):1 mm, (i-l): 100 µm.

6.3.2 Chitosan/PRP implants solidified quickly *in situ* and improved the macroscopic repair appearance in chronic defects

Clear differences in the solidification and stability of implants were observed at the time of surgery. On average, CS/PRP implants solidified *in situ* within 30-60 seconds. In contrast, in most cases, recalcified PRP implants did not coagulate even after passage of 5 minutes. In general, assessment of 8 week repair outcome was found to be generally poor [Fig. 6.3], most likely due to the chronic nature of the current defects. Incomplete fill and poor repair tissue integration to adjacent cartilage tissue were observed in both groups [Fig. 6.3]. Defect surfaces were significantly depressed with irregular surface [Fig. 6.3]. Appearance of repair tissues varied from dense white, glossy to reddish, spongy or tufty [Fig. 6.3]. Although higher for BMS+CS/PRP group (Mean score 4.75 ± 2.25) compared to BMS+PRP group (Mean score 3.25 ± 2.05), macroscopic ICRS scores did not show a significant difference between treatments (p=0.16) [Fig. 6.3e].

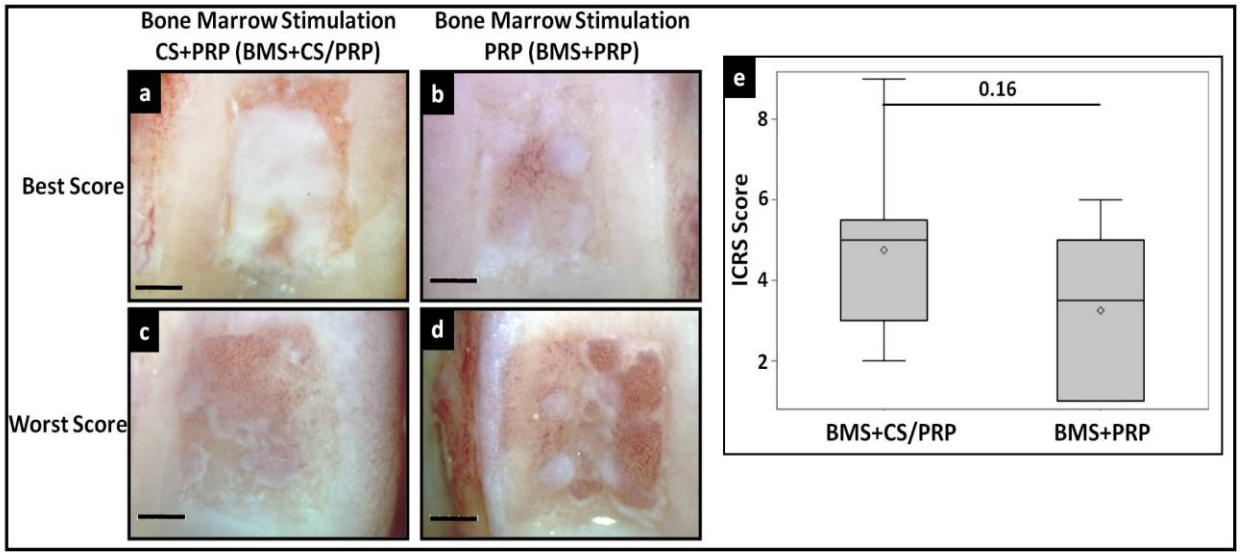


Figure 6.3: Best (a,b) and worst (c,d) repair response in defects treated with BMS+CS/PRP (a,c) and BMS+PRP (b,d). Scale bar=1 mm. (e): Mean macroscopic ICRS score was higher (non-significant) in defects treated with BMS+CS/PRP versus defects treated with BMS+PRP.

However, the BMS+CS/PRP group had the only instance of a nearly normal (grade II) repair response and only two severely abnormal (grade IV) repair outcomes, while the BMS+PRP group had 4 instances each of abnormal (grade III) and severely abnormal (grade IV) outcomes [Table 6.1].

Table 6.1: Number of defects in each repair category for both treatments. Macroscopic repair scored according to the ICRS system.

Grade of repair	BMS+CS/PRP	BMS+PRP
I (12-Normal)	0	0
II (8-11 Nearly Normal)	1	0
III (4-7 Abnormal)	5	4
IV (1-3 Severely Abnormal)	2	4

6.3.3 Histological assessment showed superior repair in defects treated with chitosan/PRP implants

Higher expression of GAGs and type II collagen was observed in defects with the best histological scores [Fig. 6.4 a,b,i,j,e,f,m,n], compared to defects with lowest scores [Fig. 6.4 c,d,k,l,g,h,o,p]. Where present, repair tissues showed good integration to underlying bone, although bonding with adjacent cartilage was poor in both groups [Fig. 6.4].

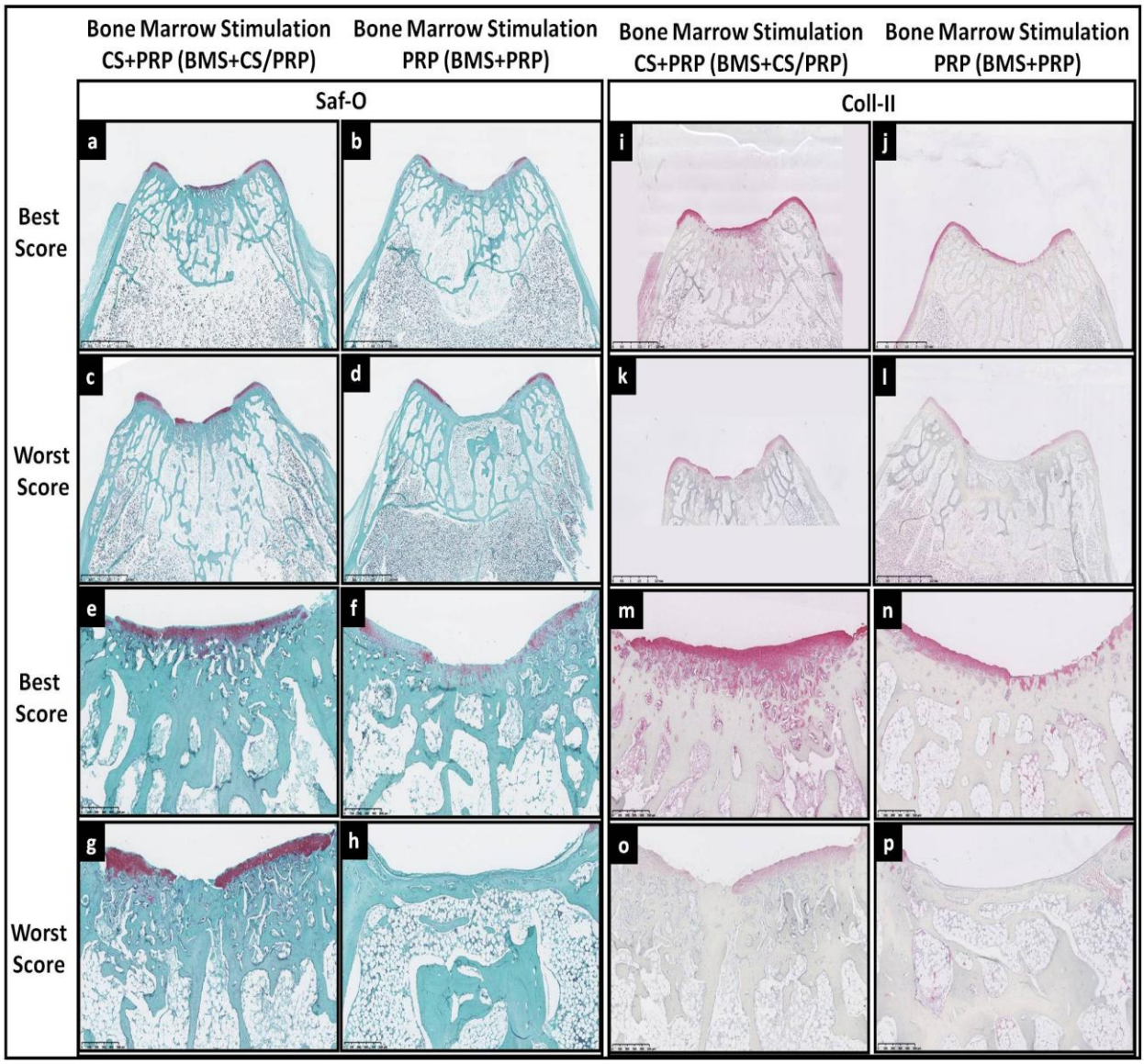


Figure 6.4. Comparison of histopathological assessment of best and worst repair tissues generated by BMS+CS/PRP and BMS+PRP. **(a-h)**: Saf-O staining for best (a,b,e,f) and worst (c,d,g,h) repair outcomes; **(i-p):** Coll-II immunostaining for best (i,j,m,n) and worst (k,l,o,p) repair outcomes; Scale bars 2.5mm (a-d & i-l) and 500 µm (e-h & m-p).

Restoration of surface, structural integrity and thickness were all improved by BMS+CS/PRP treatment (p=0.05, p=0.0001 and p=0.002, respectively) [Fig. 6.5 a,b,c,d] and [Fig. 6.6 b]. Hypocellular tissue was less frequently observed in BMS+CS/PRP group and incidence of

chondrocyte clustering was more frequent in the BMS+PRP group (p=0.002 and p=0.009, respectively) [Fig. 6.5 g,h,i,j] and [Fig. 6.6b]. Margins of defects were recognizable and degenerative changes were observed in the adjacent cartilage, especially in the BMS+PRP group (p=0.004) [Fig. 6.5 e,f] and [Fig. 6.6 b]. Zonal organization and tidemark were not restored in any defect at this 8 week time point [Fig. 6.5 g,h,i,j]. One case of a cleft communicating with subchondral cyst was observed in both groups (Data not shown). Taken together, mean O'Driscoll score was significantly higher for BMS+CS/PRP group (Mean score 20.5±1.69) versus BMS+PRP group (Mean score 14.75±1.75) (p=0.0002), indicating superior quality of repair in presence of CS/PRP implants [Fig. 6.6a].

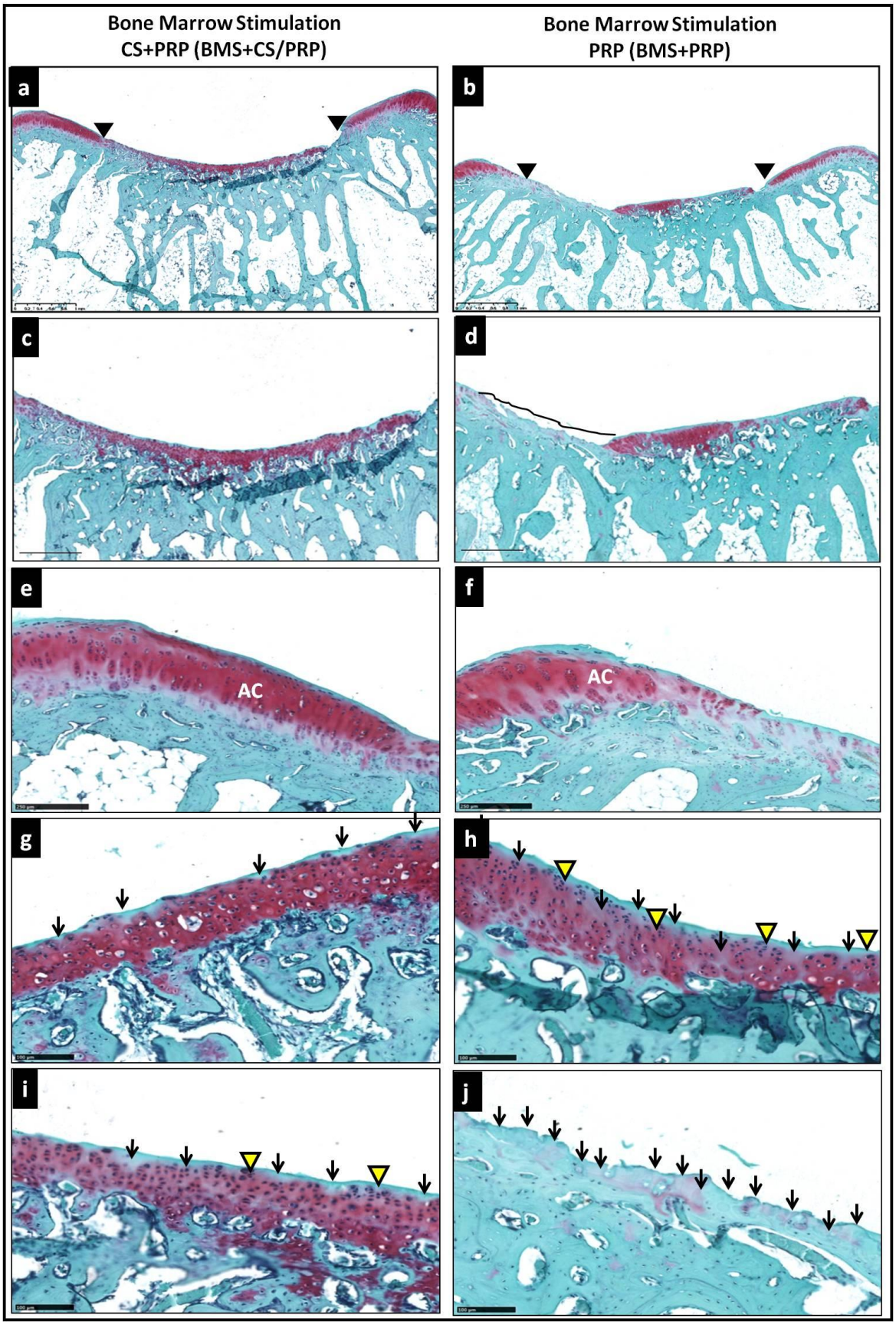
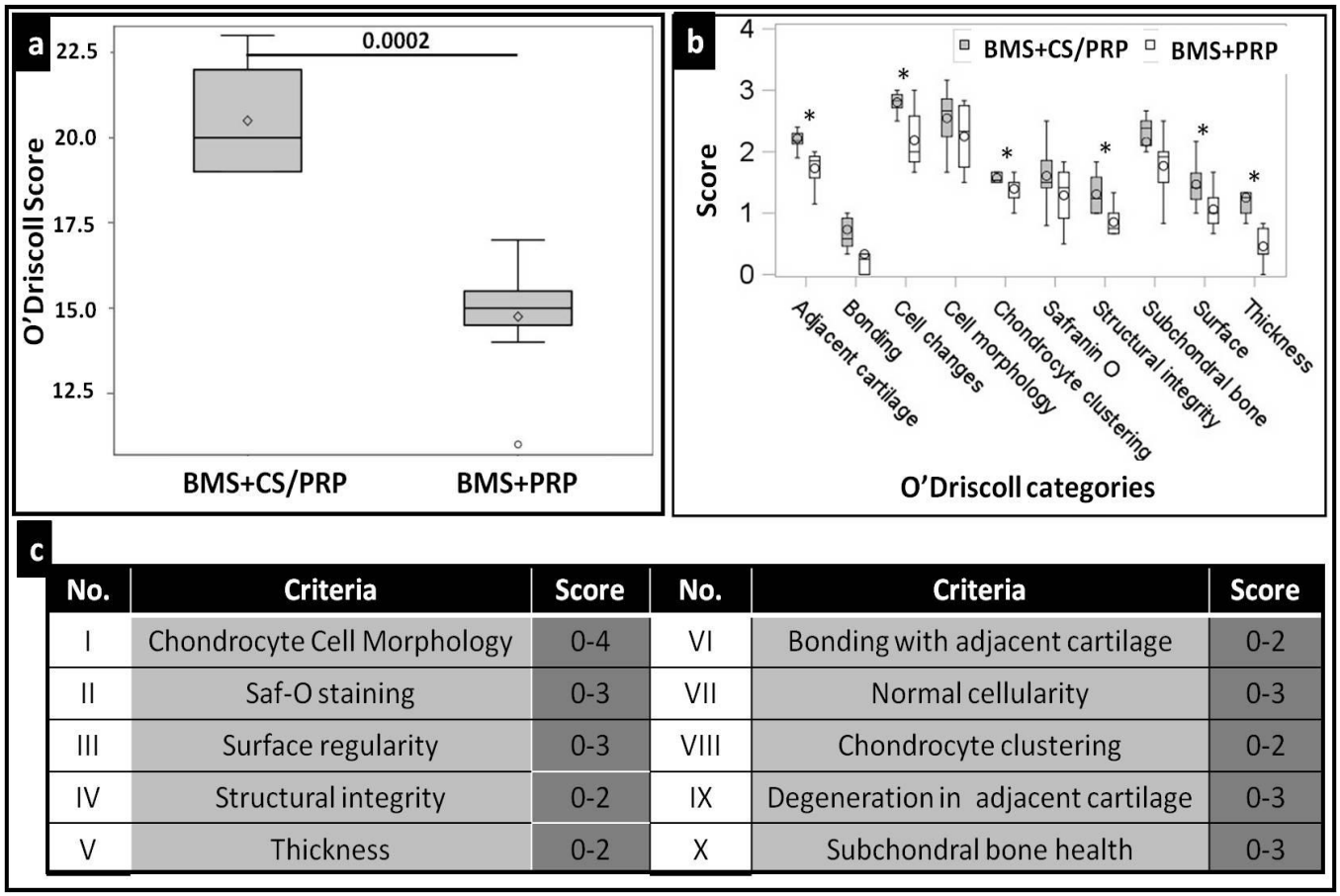

Bone Marrow Stimulation
CS+PRP (BMS+CS/PRP)
Bone Marrow Stimulation
PRP (BMS+PRP)
a
b
c
d
e
AC
f
AC
g
h
i
j

Figure 6.5: Representative sections of repair tissues generated by BMS+CS/PRP and BMS+PRP. (a,b): Restoration of surface and structural integrity was better in presence of CS/PRP (a) versus PRP (b) (defect margins flanked by solid black arrows); (c,d): Missing repair tissue (line) in BMS+PRP (d) versus more uniform tissue in BMS+CS/PRP (c); (e,f): Comparison of adjacent cartilage (AC) showing improved appearance in the case of BMS+CS/PRP; (g,h): Best sections, (i,j): worst sections- all sections from same animal. Black arrows indicate zones of hypocellularity, yellow arrows indicate cell clusters, both more frequent in BMS+PRP. Scale bars=a,b: 1 mm, e-f: 250 µm, g-j: 100 µm.



c

No.	Criteria	Score	No.	Criteria	Score
I	Chondrocyte Cell Morphology	0-4	VI	Bonding with adjacent cartilage	0-2
II	Saf-O staining	0-3	VII	Normal cellularity	0-3
III	Surface regularity	0-3	VIII	Chondrocyte clustering	0-2
IV	Structural integrity	0-2	IX	Degeneration in adjacent cartilage	0-3
V	Thickness	0-2	X	Subchondral bone health	0-3

Figure 6.6: (a) Mean O'Driscoll score was significantly higher for repair tissues in defects treated with BMS+CS/PRP versus defects treated with BMS+PRP. (b). Significant differences (*) were observed between treatments, and scores for adjacent cartilage (p=0.004), cellular changes (p=0.002), cell clusters (p=0.009), structural integrity (p=0.0001), surface integrity (0.05) and

thickness of repair tissue (p=0.002) were significantly higher for defects treated with BMS+CS/PRP. (c). Criteria used in modified O'Driscoll scoring with respective score range.

Quantitative histomorphometry revealed a significant increase in type II collagen staining in repair tissue matrix for BMS+CS/PRP group (Mean 57.37±12.43) compared to BMS+PRP group (Mean score 32±15.94), indicating a more hyaline repair (p=0.003) [Fig. 6.7a]. Safranin O staining was less widespread than type II collagen staining [Figs. 6.4, 6.5, 6.6]. A higher proportion of repair tissue was GAG-positive in the BMS+CS/PRP group (Mean 44.9±16.3) compared to BMS+PRP group (Mean 34.6±8.9), although this result was not significant (p=0.07) [Fig. 6.7b].

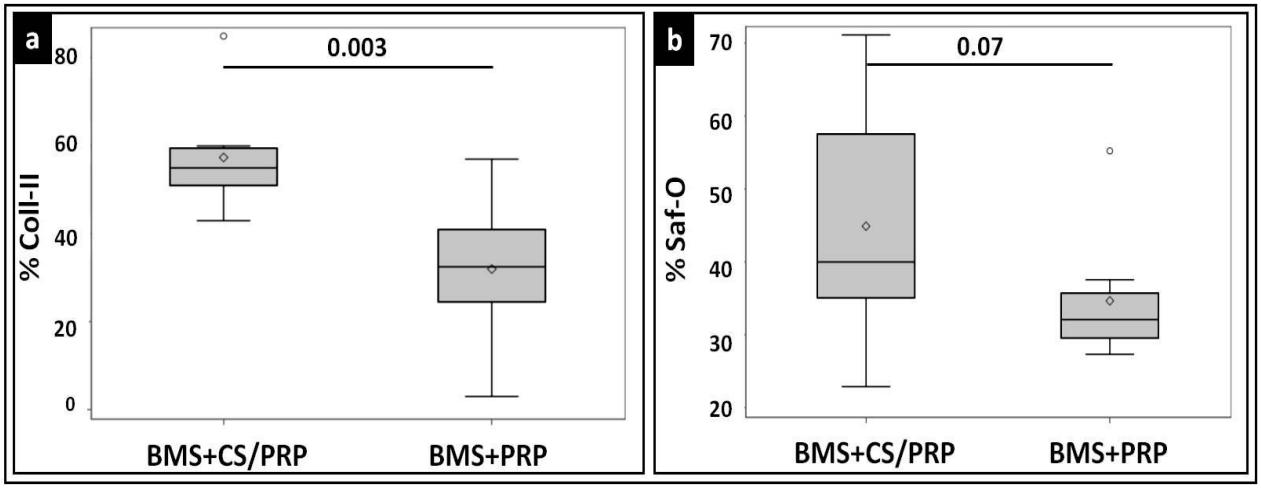


Figure 6.7: a). Mean %Coll-II was significantly higher for repair tissues in defects treated with BMS+CS/PRP versus defects treated with BMS+PRP. b). Mean % Saf-O was higher for repair tissues in defects treated with BMS+CS/PRP versus defects treated with BMS+PRP, although this difference was not significant.

6.3.4 Chitosan/PRP implants induced bone remodeling in BMS-treated defects

In both groups at 8 weeks post-operative, subchondral bone underneath cartilage defects showed evidence of ongoing remodelling indicating repair was still underway [Fig. 6.4]. Quantitative 3-D micro CT analysis revealed high inter-individual variability in bone structural parameters, and no significant difference between treatments [Fig. 6.1S]. However, the values for bone surface density, bone surface, connectivity density, and trabecular number were all higher for BMS+CS/PRP group compared to BMS+PRP group (all $p=0.01$), indicating that CS/PRP implants increased bone remodeling [Fig. 6.1S].

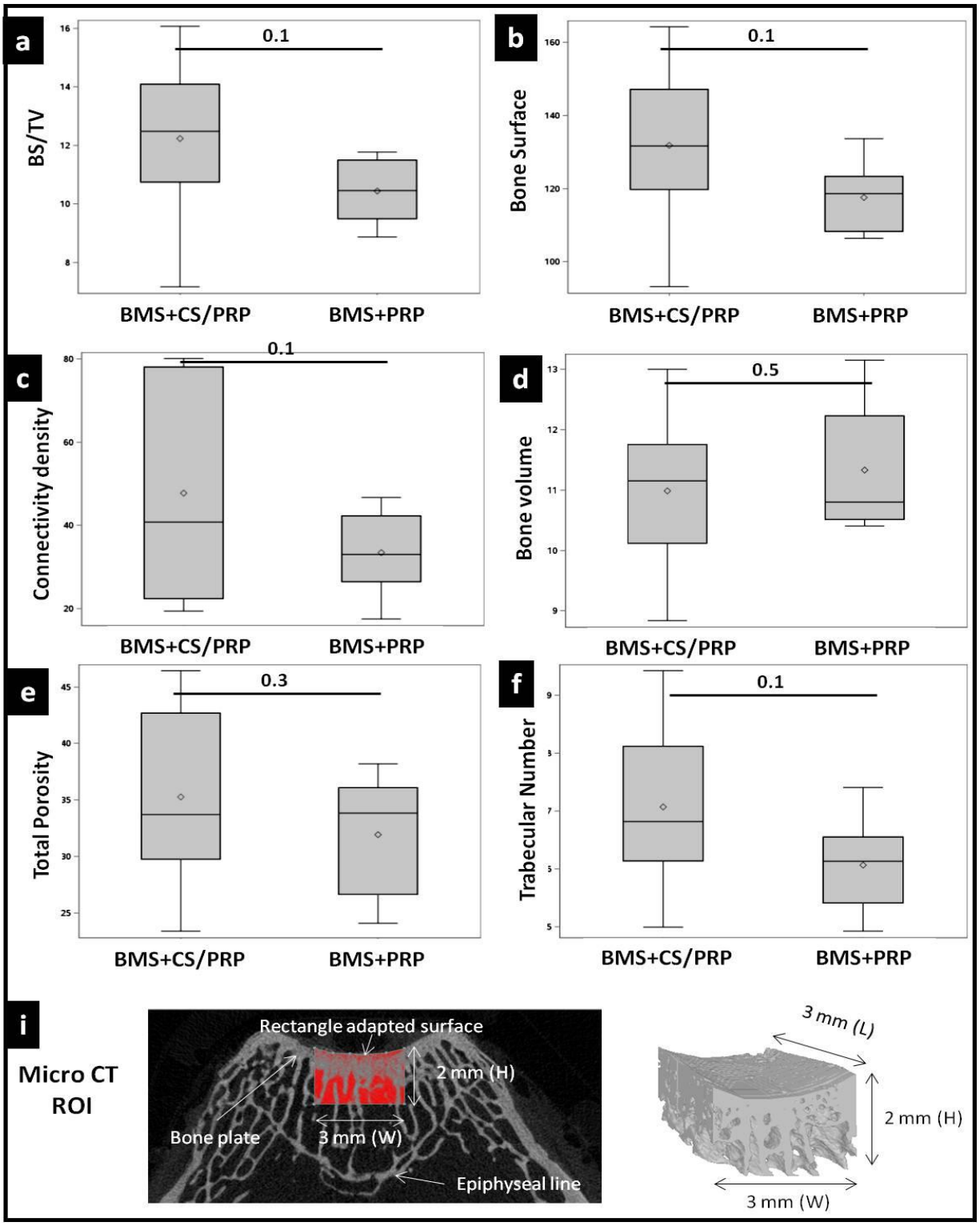


Figure 6-1S: MicroCT 3-D analysis showed differences in structural parameters between defects treated with BMS+CS/PRP versus defects treated with BMS+PRP. Although the results were not significant, the values for bone surface density (a), bone surface (b), connectivity density (c), and

trabecular number (f) were trending high for BMS+CS/PRP group versus BMS+PRP group, suggesting an increase in bone remodeling. i. Schematic representing the region of interest (ROI) for micro CT analysis.

6.4 Discussion

A chronic model was developed to test the efficacy of CS/PRP implants in augmenting marrow stimulated repair. Although chronic defects were found to be more challenging to treat than acute defects, CS/PRP implants improved the quality of repair tissues, which suggests that they would constitute a promising approach for treating chronic, degenerated lesions in older patients. Taken together, our data shows superiority of BMS+CS/PRP in repair of chronic defects compared to BMS+PRP, thereby affirming our starting hypothesis.

The chronic defect model used here was intended to approach the degenerative and inflammatory processes concomitant with metabolic alterations in early OA due to cartilage injury. In line with this, we found that BMS by drilling to 6 mm induced much poorer repair in trochlear chronic defects [Figs. 6.3,6.4] than in similar acute defects in the rabbit model [34,35]. Recent studies have reiterated the chronic defect model to be more suitable to study pathogenesis of OA which is associated with multiple changes in the defect milieu with severe bearing on downstream repair processes. Altered joint homeostasis in old defects has been associated with inferior repair [40]. The difficulty in treating chronic defects has been recognized in multiple studies in the past [40,41]. Saris *et al* showed that cartilage repair outcome in groups receiving late treatment was significantly inferior compared to early treatment group and was comparable to untreated group [40]. Rodrigo *et al* suggested that synovial fluid may have a stimulatory effect in treatment of acute defects while may be inhibitory in chronic defect treatment [42].

PRP is a rich source of growth factors and cytokines which play important roles in inflammatory and wound repair phenomena [9,10]. Although the exact mechanism of PRP action has not been understood, BMSC recruitment, expression of cartilage matrix [18] in addition to migration, proliferation and chondrogenesis of subchondral BMSCs have been shown to be positively influenced by PRP. In contrast, some studies report that PRP exerts a stimulatory effect on proliferation of chondrocytes and BMSCs, but has no effect on their chondrogenic differentiation [17, 47-49]. Reduced expression of lineage specific markers was observed in cells expanded in the presence of PRP [50]. Therefore, we believe that BMS augmented with recalcified PRP was

an appropriate baseline treatment to be compared with BMS repair outcome elicited in presence of CS/PRP implants. Our results indicate insufficient capability of PRP in enabling cartilage healing in a chronic defect [Figs. 6.3,4,5,6,7]. Similarly, in a previously published study, PRP was unsuccessful in regenerating the hyaline nature of repair tissue in a chronic defect treated with BMS [18]. In our study, PRP failed to solidify even after several minutes in most cases, suggesting impaired *in vivo* residency of a liquid implant, and providing a possible mechanism for reduced efficacy of PRP in cartilage regeneration. In our recent study, CS/PRP clots showed significant increase in viscosity vs PRP and reduced clotting time by 4 times compared to recalcified PRP [23]. Whereas CS/PRP clots remained voluminous even 1 hour after clotting, recalcified PRP clots had lost ~80% of their original volume as a result of serum exudation [23]. The resulting quick loss of platelet-derived bioactive factors could potentially limit repair in these defects. In our implants, CS inhibited the retraction observed in PRP clots and solid, voluminous CS/PRP implants increased stability *in vivo*, ensuring bioactivity for several weeks. Consistent with this hypothesis, CS/PRP implants have been shown to reside for several weeks *in vivo* and to possess significant bioactivity while recalcified PRP degraded in a day [23]. In addition, when implanted subcutaneously, the sustained presence of CS/PRP implants induced cell recruitment and angiogenesis in the local milieu. A similar increase in recruitment of progenitor cells combined with increased angiogenesis will greatly influence the cartilage tissue regeneration and subchondral bone remodeling. Possibly through platelet activation, CS increases the concentration of bioactive factors including proangiogenic factors [61]. Our own in-house preliminary data [62] and other studies suggest that CS may stabilize platelets in PRP leading to sustained release of GFs [58]. Kim *et al* had shown improved chondrocyte proliferation and matrix synthesis induced by sustained release of TGF-βI from CS scaffolds [63]. Taken together, we believe that superior physical stability of CS/PRP implants leads to a significant increase in their biological activity arising from sustained residency and release of platelet-derived growth factors and inherent benefits of CS.

Potentiating effects of CS in articular cartilage healing has been demonstrated in multiple studies [51-54]. While CS helped in maintenance of morphology and ECM synthesis by chondrocytes *in vitro* [53,54], a thermosensitive CS gel promoted chondrogenic differentiation of BMSCs [55].

CS has been shown to be chemotactic and increase recruitment and proliferation of BMSCs into defect [4], impede loss and preserve viability of BMSCs [56]. Three-week analysis of repair tissues in our model revealed the presence of vascularized granulation tissue enriched with polymorphonuclear cells along with enlarged drill holes in CS/PRP group, similar to what was previously reported for CS-GP/blood implants [4,57]. In contrast, a typical fibrocartilagenous repair and endochondral ossification associated with hypertrophied chondrocytes and vascular invasion was observed in BMS only group, as previously reported [1, 4]. CS-GP/blood implants improve cartilage repair by, in part, increasing cell recruitment, vascularization and bone remodeling, polarizing the macrophage phenotype towards the alternatively-activated pro-wound healing lineage and stimulating secretion of anabolic wound repair factors [4, 5, 58-60]. Wound bloom effect as seen in previous studies was characterized by enhanced woven bone plate repair in the drill holes along with increase in volume and hyaline quality of cartilage tissue, likely due to increased recruitment of chondrogenic stem cells to the cartilage lesion [57]. Here, we showed that CS/PRP implants appear to induce similar mechanisms. Taken together, these factors may drive the superior repair response in presence of CS/PRP implants indicated by more hyaline nature of repair accompanied by better macroscopic regeneration and increased bone remodeling.

One limitation of this study was its relatively short 8 week time point. Although increased hyaline nature of the repair tissue obtained with BMS+CS/PRP would be expected to provide long-term durability, this was not assessed here. In addition, we found high inter-individual variability in our results, which may arise due to immediate load bearing [64] further compounded by small sample size used in this study. Nevertheless, in spite of these limitations, CS/PRP implants show promise in augmenting the beneficial effects of BMS in chronic models.

6.5 Conclusions

We believe that this study will motivate earnest reassessment of pre-clinical cartilage repair models. Since prompt diagnosis and treatment of cartilage defects is rare, approaches aimed at improving repair in presence of altered joint hemostasis is warranted. The chronic model studied here is more comparable to human chronic defects than the corresponding acute model. Our

results show promising results in unveiling the positive role of CS/PRP implants for improvement of BMS mediated cartilage repair in chronic defects. In the future, a larger study with increased duration of repair and additional control groups will be undertaken to shed more light on the underlying mechanism of inflammatory and metabolic changes accompanying pathogenesis and repair of chronic defects using CS/PRP implants.

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CHAPTER 7 GENERAL DISCUSSION

This study was carried out with a purpose to identify the underlying mechanism of variability in marrow stimulated repair outcome by identifying location-, age- and donor-dependent factors influencing the inherent properties of subchondral bone progenitor cells (or bone mesenchymal stem cells, BMSCs). Furthermore, efficacy of a novel biomaterial implant based approach in improving repair was tested in a challenging, degenerative cartilage lesion. Our initial hypothesis was that *in vitro* analysis of inherent properties of BMSC - such as number, clonogenicity and differentiation potential - will be influenced by location and age. Results showed that rabbit trochlear vs. condylar subchondral bone yielded a greater number of progenitors with superior cartilaginous matrix expression under chondrogenic conditions suggesting higher intrinsic capacity for cartilage repair compared to condylar subchondral bone. These properties declined with increasing age. Our second hypothesis was that inherent properties of progenitor cells are the underlying cause for donor and location dependent variability in cartilage repair outcome. Quality of trochlear repair was superior compared to condyles and correlated with number, clonogenic and differentiation potential of the stem cells analysed *in vitro* in a location- as well as donor-dependent manner. We also report data in support of third hypothesis that marrow stimulated cartilage repair will be improved when augmented with Chitosan and PRP in a skeletally mature, chronic defect model. The aggressive chronic model studied here was clearly a challenge to treat and closely depicted the clinical situation. When compared to PRP alone, Chitosan/PRP implants were found to be a promising approach to address the demanding and hard to treat cases including chronic, degenerative lesions in skeletally mature donors.

Based on our data, number of BMSCs at the time of isolation is likely to exert the most significant influence on their cellular behaviour *in vitro* and a subsequent impact on the downstream differentiation process. Fewer BMSCs were isolated from condyles in young, skeletally mature and old animals. Since close interactions of cells is a critical requirement for chondrogenic differentiation of BMSCs, a lower population of these cells in condyles will likely attribute to reduced chondrogenesis eventually leading to poor repair. Evidence also suggests that paracrine effects of BMSCs are implicated in their therapeutic role [82]. Depleted BMSC population would diminish their beneficial paracrine effects and ultimately reduce their regeneration and repair potential. Stromal cells have also been shown to express angiogenins

which aid in bone remodelling due to their role in vessel growth, capillary proliferation and expansion of sinusoidal spaces [282]. In addition, they act to increase the local stem cell population by stimulating proliferation of tissue specific stem cells and inhibiting local apoptosis [282]. Since our current model engages lesser condylar stroma, both bone remodelling and stem cell proliferation may be negatively affected leading to inferior repair outcome in condyles. GLM analysis of biological properties enabled us to recognize that rabbit repair outcome is significantly influenced by resident population in subchondral bone and likely leads to location-dependent variability. *In vitro* and *in vivo* variability observed in trochlea further strengthened our conclusion. A strong correlation between cell yield of trochlear BMSCs, surface marker expression, chondrogenic differentiation potential and repair outcome was unmistakably evident in poor trochlear responders. In addition, a significant reduction in progenitor cell population was observed in older animals. Although we did not study cartilage repair in an old animal, inferior cartilage repair outcome is a well known phenomenon in older patients. Age-related reduction in progenitor cell population possibly arises from senescence. It is highly likely that reduced progenitor cell population leads to inferior repair outcome in older individuals.

Clonogenic potential of BMSCs demonstrated significant correlation with location of defect and age of donor. Colony forming potential of BMSCs determined by means of CFU-f assay is one of the parameter used to assess the stemness of a BMSC population and has been shown to have a direct bearing on differentiation potential. Trochlear BMSCs were consistently associated with a significantly higher colony forming potential indicating superior clonogenic potential. Multiple studies correlate the colony forming potential of BMSCs with multilineage differentiation potential. However, BMSC cultures are known to be highly heterogeneous and are prone to be influenced by method of isolation, culture conditions, media composition and passage number [77]. Inter-individual variability observed in poor vs good trochlear responders might also arise from the inherent heterogeneity of BMSCs, the exact mechanism for which is still poorly understood. Structural and anatomical differences along with ontogeny-dependent factors might contribute to variability in phenotypic profile of BMSCs studied under relatively similar conditions. In an important finding by Muraglia *et al*, it was observed that contrary to popular belief, only a fraction of BMSCs are indeed tripotential with capability to differentiate into osteogenic, chondrogenic and adipogenic lineage. Remaining cells are uni- and bipotent cells with much more defined propensity for a certain lineage [100]. Moreover, a difference in the rate

of proliferation between the clones was noted in further studies. Russell *et al* found that tripotent clones had higher rate of proliferation accompanied by a lower rate of apoptosis compared to unipotent clones [127]. Therefore, a difference in the proportion of tripotent clones would not only affect the differentiation potential but also cell number, both of which are expected to have a profound impact on the repair potency. Finally, cells may not be sensitive to inducing agents because of being in different stages of lineage progression [376]. Taken together, caution is required while analysing the colony forming potential of BMSCs as an indicator of differentiation potential of an individual cell.

In addition to cell number and clonogenic potential, cell surface marker profiling was also carried out. An earlier study comparing the characteristics of stem cells isolated from different niches found mild location-dependent differences in their surface marker profile and differentiation potential. The authors believed the differences to stem from the influence of the local environment the cells are exposed to in their niche [119]. Donor-dependent correlation between cell surface marker expression and biological properties was identified in poor vs good trochlear responders, thus substantiating our understanding that inherent properties of BMSCs including their marker profile is likely to impact the corresponding repair potential to a certain extent. However, location-dependent variability in the cell surface marker profile was not evident. This observation might be an indicator of limitation of *in vitro* cultures in being a true reflection of the phenotypic profile of BMSC as found *in situ*. It is hard to ascertain if the marker profile was altered as result of *in vitro* culture since expression of markers is highly sensitive to culture conditions [282].

Several factors contribute to the differentiation potential of BMSCs *in vitro* and *in vivo*. Even though rabbit MSCs (rbMSCs) and human MSCs (huMSCs) have been shown to bear close resemblance [100], rbMSCs might take longer for differentiation and have a greater propensity towards chondrogenic differentiation compared to huMSCs. Spontaneous repair potential and high inter-individual variability in rabbit repair models is a well known phenomenon. A study which directly correlates the properties of stem cells and *in vivo* repair outcome in an animal model might be the next step in understanding of pre-existing differences at the two locations. Characterization of BMSCs isolated from young trochlea and condyle revealed high inter-individual variability. Differences in the surface receptor profile, interactions with growth factors

and cytokines, stages of lineage progression, epigenetic factors and posttranslational modifications are some of the factors likely implicated in the inherent variability of BMSC population [58]. The downstream differentiation commitment of BMSCs may also be influenced by spatial dimensionality modifying the interaction of BMSCs in trochlea and condyle and their corresponding microenvironment [377]. The outcome of differentiation assays was more consistent in skeletally mature and older individuals. This might be due to increase in number of progenitor cells with defined propensity for a certain lineage. An age related reduction in number of tripotential progenitors has been demonstrated in the past [378]. Studies have also shown that stem cell's multipotentency is also influenced by the histone modification patterns of differentiation-associated genes [287]. A difference in these modifications arising due to anatomical differences, mechanical and chemical cues or some unknown inherent mechanism may also impact the differentiation potential of the stem cells and may lead to donor-, age- or location dependent variations [287].

In addition to stromal cells, we also carried out the characterization of cells migrating out of the bone explants. It is difficult to determine the niche of BMSCs due to lack of specific antigens or a specific phenotype. Nonetheless, they are hypothesized or postulated to be located in perivascular structures from where they can migrate to the site of need upon stimulation [379]. An alternative hypothesis in case of BMSCs is they migrate out of bone when cultured as explants over a period of time [380]. These cells are protected from the action of collagenase enzyme due to the presence of mineralized matrix and begin proliferating on being liberated later on. They are presumed to be undifferentiated/dedifferentiated cells possessing multilineage differentiation potential. They do not express any marker of bone except Col-I and are characterized by a weak expression of ALP [380]. In fact, Lin *et al* conducted an interesting experiment to show the migration of Stro-1+ cells from the bone chips to give rise to a culture of BMSCs bone chips and proposed that explants are richer source of BMSCs compared to any other source including BM. In our histological images of bone chips from day 7, presence of numerous lacunae in the bone was seen which could be a result of migration of cells or the death of osteocytes. If the cells indeed migrate out, these could revert to de-differentiate stage and regain multilineage differentiation potential. Lin *et al* provided evidence of this migration in their study indicated by reduction in percentage of Stro-1+ cells accompanied by an increase in number of lacunae in bone matrix [380]. We found remarkable similarities in most of the characteristics of collagenase

and explant derived BMSCs, providing further evidence that cells in bone have the potential to dedifferentiate into a more primitive and undefined phenotype.

In an important observation, we found a strong influence of age of BMSCs on the corresponding biological properties including their number, stemness, expression of cell surface markers and differentiation potency. Reduced migration potential due to decreased response to chemoattractants might also contribute to inferior repair outcomes in older individuals. Ageing also induces changes in chondrocytes including senescence, reduced response to growth factors, accumulation of AGE and oxidative damage [287]. The resultant disturbance in the cartilage homeostasis makes the cartilage matrix vulnerable to further damage. Reduction in cell number and senescence in existing progenitor cell population are prevalent in older individuals. In addition to poor telomerase activity, the genes involved in DNA replication, cell cycle, mitosis and DNA repair are significantly downregulated with increasing age [381]. As a result of accumulation of DNA damages, cells enter senescent phase. According to Hayflick model of senescence, once the cells reach senescent limit, they switch from a rapidly dividing phenotype to a slow dividing one accompanied by a loss in differentiation potential [100]. There is a decline in anti-oxidants with increasing age which leads to escalation of levels of reactive oxygen species [382]. Moreover, the levels of growth factors decrease which might cause a shortfall in factors required critically for differentiation. Finally, Xin *et al* proposed that migratory ability of BMSCs towards chemoattractants reduces with increasing age [383]. This is an important consideration since it might lead to reduced migration and recruitment of BMSCs towards cartilage lesion in older individuals. The data from our study is consistent with the earlier findings of Payne *et al* and Zheng *et al* where a reduced chondrogenic potential was observed in older rats [220, 221]. This was confirmed in chondrogenic and osteogenic differentiation assays. Older animals had negligible Col-II and Saf-O and Alizarin Red-S staining denoting poor cartilaginous and osseous matrix. On the other hand, BMSCs from young animals synthesized a matrix rich in cartilaginous and osseous proteins.

In situ characterization of BMSC is currently lagging due to several challenges such as the lack of markers for their *in vivo* identity as well as markers to distinguish multipotent BMSCs and committed progenitor cells. As a result, it becomes imperative to draw information from the *in vitro* cultures of BMSCs. However, the conditions *in vitro* are far different from the *in vivo* niche

of BMSCs. Moreover, *in vitro* cultures are vulnerable to be affected by the heterogeneous nature of BMSCs [120]. Nonetheless, *in vitro* characterization of BMSCs is our best bet to understand the mechanism of inconsistency in the cartilage repair response and deliberate the approaches to address them. Our second study (chapter 5) aimed to provide further understanding of underlying mechanism of inherent variability in cartilage repair response, thus bringing us one step closer to developing approaches to reduce the unpredictability in marrow stimulated repair outcomes. Cartilage repair does not occur in isolation and is bound to be influenced by factors generated by defect milieu including synovium, synovial fluid and subchondral bone. Therefore, our bilateral model aimed at parallel study of repair outcome and properties of BMSCs was a more ideal approach for a better understanding of correlation between *in vivo* repair outcome and *in vitro* biological properties of BMSCs. Such a model would also be more suitable for enhancing our understanding of heterogeneous behaviour of BMSCs which might explain the influence of donor, age and location on repair outcome. A better understanding of mechanism of inter-individual variability will likely enhance predictability of MS repair outcomes. Although multiple studies have reported correlation of biological properties of BMSCs and their impact on repair outcome, our study was the first to provide valuable insight into mechanism of inter-individual variability observed in marrow stimulated repair outcome. We found direct evidence of a correlation between number, clonogenic potential and differentiation potential of BMSCs and healing induced by BMS. Animals of similar age and sex profile demonstrated clear differences in repair outcome and properties of BMSCs as a function of both location and donor. While this may be a factor contributing to the reduced differentiation potential of older BMSCs, it might also add to reduced variability between BMSC populations. By applying suitable statistical model, we were able to recognize the factors with maximum impact on the cartilage repair outcome in a location- and donor-dependent manner. Macroscopic appearance of repair tissues showed strongest association with clonogenic (CFU) & early chondrogenic potential (Coll-II) of BMSCs. O'Driscoll scores in repair tissue were most strongly related to clonogenic (CFU) & matrix production (Saf-O and Coll-II) by BMSCs. %Saf-O &% Coll-II in repair tissues were most strongly associated with cell number (cell yield) & early chondrogenic potential (Coll-II) of BMSCs. As a result, cell number, clonogenic and chondrogenic potential of BMSCs were identified as strong predictors of macroscopic and histological outcomes in rabbit cartilage repair. In conclusion, the pre-existing properties of progenitor cells may have a critical role in

influencing donor- (good vs poor responders in trochlea) and location-dependent (condyle vs trochlea) variation observed in marrow stimulated repair outcome.

Several groups are working towards challenging the limitation of BMS in older individuals with chronic, degenerative lesions. Based on our characterization studies we recognized that cell number and inherent differentiation potential of subchondral progenitor cells has a critical impact on the repair outcome. Moreover, differentiation of BMSCs is likely to be influenced by changes in joint capsule, composition of matrix and interaction with bioactive factors released by synovial fluid and subchondral bone [384]. Studies have shown that the migratory ability of BMSCs is affected by the receptors and adhesion molecules expressed by them including chemokine receptor type 4 (CXCR4) and its binding protein stromal-derived factor 1-α (SDF-1α) [285]. The expression of these mediators is vulnerable to being influenced by the changes in the environment of the BMSCs, such as presence of inflammation. Moreover, levels of MMPs are also affected by the levels of inflammatory mediators IL-1β, TNF-α and TGF-1 β thereby affecting the BMSC migration [385-387]. The chronic model used here is more likely to accurately represent the alterations induced in a degenerated joint versus the commonly used acute animal model. It has been reported that the proliferative and chondrogenic capacities of BMSCs are affected by disease stage of donor and BMSCs obtained from patients with OA are reduced compared with those from healthy individuals [53]. Chronic defects may involve altered joint biomechanics or alteration in the subchondral bone progenitor cell population thus reducing the repair potential in this model. Although no alterations were observed upon macroscopic examination of patellofemoral and tibiofemoral compartments, further investigation may reveal factors affecting the nature of repair. More specifically, changes in subchondral bone revascularization or remodelling may adversely affect cartilage repair in this rather aggressive model and need to be further explored. Since the model requires two surgeries within a short span of four weeks, it will be worth deliberating the influence of local inflammation on the efficacy of treatment strategies. In this study, we explored the beneficial effect of chitosan/PRP in improving the cartilage repair outcome.

Earlier studies have shown that application of PRP causes a significant improvement in BMSC recruitment, angiogenesis [348, 349], expression of cartilage matrix [25], proliferation and viability of chondrocytes and BMSCs [388, 389], migration and chondrogenic potential of

subchondral BMSCs [26]. However, most of the growth factors are released quickly from PRP leading to loss in their activity and clinical efficacy [305]. It has previously been shown that platelet-mediated retraction is inhibited in CS/PRP hybrid clots and that CS/PRP implants have a superior residency and bioactivity compared PRP only implants *in vivo* [390]. The observations have been described in more detail in chapter 6, article 3. The positive impact of chitosan and PRP on the recruitment, proliferation and differentiation of BMSCs is multifactorial. Chitosan is a biocompatible polycationic hemostat and promotes cell recruitment and transient vascularization, improves subchondral bone remodeling and repair [13] and escalates number and area of mature chondrogenic foci [18]. Most importantly, chitosan ensures a more sustained release and retention of growth factors in the defect milieu which has a direct bearing on the cellular behaviour of BMSCs recruited to the defect [294]. It has previously been shown that release of EGF and PDGF-AB from CS-PRP hybrid clots *in vitro* increased compared to release from PRP-only clots [391]. Moreover, chitosan can aid in overcoming the negative impact of reduced proliferation and migration observed in BMSCs of older individuals thereby generating a repair tissue with superior quality and quantity. Based on our observations in a chronic model, we believe that chitosan/PRP implants hold immense potential in improving cartilage repair outcome, especially in challenging conditions.

Inter-individual variability in repair response was prevalent. Although significant improvement in the macroscopic appearance was not observed, lesser instances of grade IV degeneration was observed in presence of chitosan/PRP implants. Integration with adjacent cartilage was poor in both groups which may be explained by aggressive nature of the defect. Evidence of degeneration was more prominent on defect edges and increased chondrocyte clustering was more prevalent in PRP group suggesting a worse repair response. Additionally, chitosan has been shown to induce superior, albeit delayed repair in previously published study [13, 19]. It is likely that repair response in poor responders may be improved over time and longer repair durations might result in better repair. While 8 weeks' time point was considered to be suitable in this preliminary study, this might still be a relatively early repair point and increased GAG deposition might be observed in a model with longer sacrifice time point. An earlier study reported improved repair outcome in 26 weeks compared to 13 weeks in a chronic partial thickness rabbit model [392]. Moreover, any growth factors or cytokines released after initial injury will not have any lasting effect by the time of second surgery. Although the meniscus appeared normal, there may be

microscopic changes with profound impact on cartilage repair since cartilage degeneration has been found to be concomitant with meniscus degradation in humans. Besides, despite our best efforts, the complete removal of calcified cartilage cannot always be ensured and the presence of residual CC has been shown to be one of the barriers in marrow stimulated cartilage repair. There is also an immediate load bearing in animals with no physiotherapy during recovery phase. Appropriate physiotherapy and gradual increase in weight bearing may also improve repair outcome in humans. Finally, although this model is aimed at closely simulating advance stages of OA, an accurate reflection of alterations in matrix metabolism and accompanying cartilage degeneration seen in OA may not occur. In the light of these factors, it is reasonable to propose that selective modifications in the future will likely generate even more promising preclinical results with application of chitosan and PRP implants, thus paving way for successful clinical translation.

CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

The immense potential of BMS in cartilage repair is well recognized; even with certain limitations including poor quality of repair and high-inter individual variability. Despite several pre-clinical and clinical studies, a clear identification of source of strong age dependence and significant inter-individual variability in BMS remains unclear. This thesis presents a method of comprehensive analysis of properties of subchondral bone progenitor cells. Intricate techniques for isolation of progenitor cells from condyles and trochlea were optimized followed by their in-depth analysis using *in vitro* biological assays. Progenitor cells originating from marrow spaces and cortico-spongious bone showed mesenchymal stem cell phenotype as defined by ISCT guidelines for stem cell characterization. Rabbit trochlea yielded a greater number of progenitors with higher clonogenic and osteogenic potential versus condyle. A greater manifestation of cartilaginous phenotype was confirmed by superior cartilage matrix production in trochlea. The mechanism for significant inter-individual variability in chondrogenic potential of young animals warrants further perusal. In contrast, subchondral progenitor cells isolated from distal femur of old animals were characterized by significant reduction in yield, clonogenic and surface marker expression. Combined with significantly reduced chondrogenic potential, these observations point towards a strong age-dependent decline in properties of progenitor cells making older individuals more susceptible to unsuccessful cartilage healing. Taken together, our observations led us to conclude that inherent biological properties of progenitor cells are prone to be influenced by their location and age of individual. These observations motivated us to further verify if superior inherent properties of trochlear progenitor cells are the likely cause of improved repair in rabbit trochlea.

Since BMS procedures rely on cellular behaviour and downstream differentiation of BMSCs for cartilage regeneration, it is highly likely that pre-existing properties of progenitor cells may have a critical role in age-, donor- and location-dependent variation observed in the repair outcome. It is important to bear in mind that rabbit repair procedures are extremely sensitive to inter-individual variability and notable spontaneous repair potential. With these issues in mind, skeletally mature rabbits with more consistent repair outcomes were used to develop bilateral models. We designed a study to investigate the dependence of inter-individual variability on

location- and donor-dependent variables. Subchondral bone parameters in one knee of each animal was correlated with marrow stimulated repair capacity in second knee from same animal. Full thickness defects were created in condyle and trochlea of one knee and treated by microdrilling to induce marrow stimulated repair. Bone marrow stimulation induced superior repair in trochlea with tangible evidence of inter-individual variability leading us to recognize good and poor trochlear responders. Negligible repair tissue volume was observed in all eight condyles. ICRS score indicated significantly higher macroscopic restoration in trochlear defect. Histological and histomorphometrical examination provided further evidence of superior repair in trochlea indicated by significantly higher GAG and collagen content of matrix in repair tissue. *In vitro* characterization of progenitor cells isolated from contralateral knee confirmed our earlier findings and trochlea exhibited increased number, clonogenic potential and chondrogenic potency compared to condyles. In an important finding, we showed that the intrinsic difference in the repair potential of trochlea may be a result of lower number, reduced surface marker expression and low clonogenic and chondrogenic potential of progenitor cells present in metaphyseal marrow. We believe that reduced cell number with lower clonogenic potential causes a reduction in the downstream differentiation potential *in vitro* as well as *in vivo*. Linear regression analysis of univariate scatter plots revealed moderate to strong positive correlation between repair outcome parameters as dependent variables and biological properties as independent variables. GLM analysis with forward selection of variables enabled us to identify cell number, clonogenic and collagen expression of trochlear cells as most important variables in predicting repair outcome in an individual. These studies have allowed us to provide substantial evidence of location-, age and donor-dependent variability in marrow stimulated cartilage repair outcome. Our extensive characterization and robust analysis protocol showed that intrinsic biological properties of subchondral bone progenitors strongly influence *in vivo* repair potential.

While our analyses of subchondral bone parameters provide new avenues to improve BMS cartilage repair, it also stressed upon the need for development of approaches for successful functional repair, especially in challenging cases. Classical and augmented BMS has been tested in several pre-clinical studies. Unfortunately, promising strategies fail to translate successfully into a clinical practice since acute defect preclinical defect models are an inaccurate representation of clinical pathology. Our studies have led us to conclude that presence of voluminous, stable and adherent blood clot is a critical factor in ensuring superior quality and

quantity of regenerated tissue. Chronic defects created in adult animals were clearly more challenging to treat and were evidently distinct from fresh, acute defects. Increased biological stimulation provided by PRP stabilized by the CS implants showed superior repair potential in treatment of chronic, degenerative lesions. We believe that increased bioactivity achieved by the application of PRP along with beneficial properties of CS in cartilage regeneration stimulated better matrix restoration in chronic defects treated with BMS and augmented with CS/PRP implants. Histological analysis confirmed an overall improvement in repair in BMS+CS/PRP knees as indicated by higher O'Driscoll scores and histomorphometric assessment. Although not significant, CS/PRP implants also increased subchondral bone remodeling compared to defects treated with BMS augmented with PRP. Taking synthesis of these observations, we believe that chitosan/PRP clots have a significant potential for use in cartilage repair because of their superior handling properties as well as their increased bioactivity due to the concentration of platelets and associated growth factors.

The comprehensive analysis of diverse factors studied in this research project reveal underlying mechanism of individual-to-individual variability in BMS repair. In addition, method to further stimulate BMS with a bioactive implant has been tested. Upon combination with structural imaging, these results may be applied in development of methods for identification of factors which influence repair and assist clinicians in modifying their treatment on a more patient relevant basis, especially in demanding cases. At this point, we realize that we have made noteworthy critical progress in the field of cartilage repair; however, these conclusions only give rise to more questions which when answered can make further contribution towards providing relief from osteoarthritic pain and discomfort.

Although beyond the scope of this research, following recommendations are worth considering in future:

1. *Biochemical and molecular analysis of BMSCs*. One of the deficits of our characterization assays was the lack of a biochemical and molecular analysis. We carried out immunohistopatholgical analysis to assess the chondrogenic potential of pellets from condyles and trochlea. While the assessment was sufficient for us to conclude superior properties of trochlea, it will be interesting to investigate the biochemical and molecular

changes occurring in temporal evolution of pellets over three week culture period. Transcriptional and translational profiling do not always concur and detailed analysis of genetic expression profile might provide valuable insights. Pellets can be harvested at multiple time points during differentiation in order to reveal the molecular mechanisms responsible for differences in cellular behaviour of cells present in different locations. While expression of Coll-II and aggrecan genes can explain the differences in the matrix composition, analysis of Sox-9 may reveal important mechanism of these differences. Biochemical analysis of spent media can further aid in determining the differences in the biochemical composition of condylar and trochlear pellets and help us advance our current understanding of location-dependent variability in chondrogenic differentiation.

2. *Characterization of huBMSCs*. Although this was beyond the scope of this project, detailed characterization of huBMSCs must be carried out in future. Differences between rbBMSCs and huBMSCs explored in the past have been discussed in chapter 7. Consequently, the results from this study provide an initial understanding of the mechanism of inter-individual variability observed in humans. Extrapolation of these results would require careful consideration of anatomical, mechanical and biophysical components of cartilage repair.

3. *Correlation studies in young animals*. Correlation of properties of BMSCs and cartilage repair outcome in young rabbits will likely be complicated due to their high spontaneous repair potential. We observed high inter-individual variability in biological properties of BMSCs isolated from young condyle and trochlea. A study aimed at correlating the repair outcome with biological properties of underlying BMSCs in a young rabbit might further elucidate the mechanism for these variations.

4. *Test the expression of Col-I.* Deposition of collagen type II and GAG indicate the hyaline nature of repair tissue and pellets. In order to understand the nature of matrix composition, it is important to study the expression of Col-I. Fibrocartilagenous repair is characterized by matrix with varying amounts of Col-I. Matrix composition of repair tissue has a critical bearing on the mechanical and structural durability of repair tissue.

We could not carry out analysis of col-I in these studies due to discontinuation of anti-Col-I antibody being used in our lab. We were unable to identify an alternative Col-I antibody that performed reliably in rabbits. Identification of new antibody epitope and optimization of histological protocol for application of new antibody are in progress. Meanwhile, we were able to derive meaningful conclusions about the hyaline nature of repair tissues generated in current studies.

5. *In depth analysis of OA pathology in chronic model*. The analysis of changes during progress of OA in the chronic model developed here was beyond the scope of this proposal. Multiple pro-inflammatory cytokines and growth factors are known to aggravate the OA pathology in chronic lesions. Several challenges with an adverse effect on success of cartilage repair in a degenerative and inflammatory environment of chronic lesion have been discussed in chapter 6 and 7. Detailed understanding will help us identify responders which can serve as targets for novel repair strategies to improve repair outcome in challenging conditions.

6. *Mechanical testing of repair tissue*. Mechanical strength is an important parameter in determination of the durability and functional behaviour of repair tissue. Studies aimed at determining the mechanical strength of the repair tissues generated with different treatments might suggest successful approaches for cartilage repair in future.

7. *Additional time points for chronic model*. We chose duration of development of chronic lesion and cartilage repair based on our understanding from existing studies. Although we find one month to be sufficient time for development of OA pathology, additional time points must be studied in order to evaluate the long-term repair outcome. More often than not, BMS leads to formation of a fibrocartilagenous repair tissue with severely compromised mechanical durability. A long term study help will likely improve the quality and volume of repair outcome and enable a more meaningful testing of its durability and stability.

8. *MFX alone group*. The study presented in this research proposal compared the effect of treatments including CS/PRP and recalcified PRP. A group treated with BMS alone was conspicuously absent. The rationale for choice of PRP alone as control treatment has been discussed in chapter 6. However, in order to improve our understanding of bioactive stimulation provided by individual components of the proposed treatment, it will be imperative to conduct a study with a control group receiving treatment with BMS alone. Now that we have provided a proof of concept, a larger study with more number of animals can be planned in future with statistically powered groups to compare all treatments.

9. *Dosage analysis of CS/PRP*. Small scale studies designed to test multiple concentrations of CS after deliberate consideration might be carried out in future, specifically to suit the needs of different animal models. We need to strike a balance between efficient cartilage repair and extensive immune response leading to formation of granulation tissue which might delay repair process.

10. *Chronic model studies in a larger animal*. Structural variations in the joint anatomy have profound impact on mechanical profile of repair tissue. Application of CS/PRP must be tested in larger animals such as sheep in order to evaluate the role of denser subchondral bone in large animal taking us one step closer for eventual clinical translation.

Scientific challenges

Variability in cellular behaviour of BMSCs during *in vitro* characterization. Earlier studies have shown that drilling deep to 6 mm (D6) improved the repair outcome in trochlea when compared to drilling to 2 mm (D2). The likely cause was that deep drilling provided access to metaphyseal marrow which is richer in progenitor cell population. As a result more cells are recruited to the defect thereby improving the repair response. However, results in the current study led us to conclude that chondrogenic potential of trochlea lower BMSCs isolated from metaphyseal marrow was inferior to that of epiphyseal trochlear BMSCs. It is likely that the superior repair with D6 arises because the volume of bone participating in repair is three times the volume of bone participating in D2. This would eventually lead to an increase in the number of cells participating in the repair thus improving the repair outcome. *In vitro* culture makes this difference inconsequential and would likely be a true reflection of inherent potential of these cells. However, trochlea lower BMSCs did demonstrate highest clonogenic potential which was not reflected in the chondrogenic potential. We observed similar results with progenitor cells isolated from young and old animals. The mechanism for this observation still remains unclear. A possible explanation can be attributed to heterogeneity in multipotency of progenitor cells. Only a fraction of progenitor cells are really tripotential while a majority of population is either uni- or bipotential. It is possible that metaphyseal marrow is richer in uni-or bipotent cells while progenitor cells in epiphyseal marrow may possess propensity towards chondrogenic lineage. Also, it is impossible to determine the influence of culture conditions on the accuracy of assays as the properties of progenitor cells have been shown to be influenced by culture conditions in a profound manner.

Challenge of bilateral model in study 2. Study 2 was designed with an aim to correlate the repair outcome with inherent properties of progenitor cells present in condyle and trochlea in order to reveal a likely mechanism of variability observed in marrow stimulated repair outcome. In order to be able to draw parallels, it was important to characterize the cells with maximum phenotypic similarity to those participating in the repair process. A bilateral model was used to correlate the repair outcome in a donor-dependent fashion and also reduce inter-individual variability. This led to a difference of three weeks between the induction of repair and characterization of cells *in vitro*. We would be remiss if we did not recognize that the

modifications induced by defect generated in one knee might have an influence on the properties of BMSCs in the contralateral knee. However the model was most suitable given our limitations. Moreover, since the repair duration was limited to three weeks, we do not expect a significant influence on the properties of BMSCs in the contralateral knee and believe that their characterization provides a close approximation of their inherent properties in native state.

Chronic model in study 3. In order to better represent the clinical situation, a chronic defect model was developed in study 3. We realized that chronic defects are certainly harder to treat and present inferior outcomes than those observed in acute defects in previously published studies. Chronic defects may involve altered joint biomechanics or variations in the subchondral bone progenitor cell population thus reducing the repair potential in this model. Although no alterations were observed upon macroscopic examination of patellofemoral and tibiofemoral compartments, further investigation may reveal factors affecting the nature of repair. More specifically, changes in subchondral bone revascularization or remodelling may adversely affect cartilage repair in this rather aggressive model and need to be further explored. Since the model requires two surgeries within a short span of four weeks, it will be worth considering the influence of these treatments on local inflammation which may in turn influence the efficacy of chitosan/PRP implants. Although the meniscus appeared normal, there may be microscopic changes with profound impact on cartilage repair since cartilage degeneration has been found to be concomitant with meniscus degradation in humans. Finally, although this model is aimed at closely simulating advance stages of OA, an accurate reflection of alterations in matrix metabolism and accompanying cartilage degeneration seen in OA may not occur.

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