TOWARDS MODULATED FEEDING FOR THE EFFICIENT AND PHENOTYPICALLY CONTROLLED EXPANSION OF ARTICULAR CHONDROCYTES

By

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ABSTRACT

Cell culture of chondrocytes, either in an experimental or industrial setting, typically rely on an invariant feeding strategy. The contribution of each component of the total expansion regime to the behaviour of the cells been expanded are not fully understood. It can be argued that to fully realise the potential of cell-based therapies, the exact contribution of the various expansion parameters to growth rate, metabolic behaviour and cell phenotype need to be intimately understood.

The studies in this thesis aim to better inform the nutritional aspects of future cell manufacturing strategy by; uncovering the contribution of critical culture parameters to growth rate and phenotype, understanding the relationship of any interactions that may exist between components and how these components may influence the culture as a function of time.

Results from the various studies demonstrated that an isolated subpopulation of chondrocytes can be sustained under serum-free conditions that promote SOX-9 transcript expression while returning growth rates similar to that found using serum-based medium. The contribution of the medium components, seed density and adhesion protein concentration to growth rate, metabolic behaviour and SOX-9,6 & 5 transcript expression were quantified using a statistically designed experiment. Two factors from the experiment, EGF and FGF-2, were taken forward and their effects on growth rate and gene expression as a function of time were investigated. The findings revealed that the exact timing of the introduction of the proteins into the culture medium had a marked impact on cell growth and gene expression.
Meet Mel. Mel was a humble man from humble origins. From Doncaster, South Yorkshire, Mel was the son of a boiler house worker and a school cook. He spent his adult life working as a quantity surveyor, a job he was very fond of. Mel loved the simple things in life from music to travel, but above all else, he loved his children. Into them, he poured all his time and wealth and for him, watching them grow made life whole. Mel fell ill shortly after the summers solstice of 2016. After a hard and painful fight, Mel found the peace he deeply deserved in the early hours of 11th September 2016 leaving behind devastated family and friends.

Mel was my dad and I count myself as blessed in this life to have had one that cared so much and gave so much, all without condition. His legacy that he passes on is the principle by which he lived his life, which, in short, reads simply

.... be kind and humble to those around you as much and for as long as you can afford to be.....

See you on the other side. All my love, Dan.
ACKNOWLEDGEMENTS

Few things in life are truly accomplished without the help of others, and the research contained in this thesis is no exception to that rule. With that in mind, I extend sincerest gratitude to my project supervisors Nick Medcalf, Karen Coopman and Rob Thomas. Their many hours of guidance, support and patience are ultimately what made the project possible. Further thanks is extended to co-researchers both past and present at the centre for biological engineering for the invaluable hints and tips passed along the way. A special mention to Dimitris Tampakis who so willingly gives his time purely for the benefit of others.

Gratitude to the Loughborough Graduate School for the personal stipend and tuition fee funding as well as thanks to the EPSRC for contributions towards lab consumables.

Finally, to my family and close friends who have been there during the bleakness. It may be a tired cliché to say that there are no words that can express my gratefulness but in this case, it is true, so, I will simply say thank you, and you have my love.
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INTRODUCTION

Injuries to articular cartilage are a particular nuisance due to the tissue’s extremely limited capacity for self-repair. Acute injury to the cartilage can result in poor mechanical function of the affected joint and be the starting point for a progressive deterioration that can result in osteoarthritis. Osteoarthritis (OA) can be characterised by the gradual loss of hyaline cartilage from the joint surface, which exposes the underlying bone resulting in poor joint function and chronic pain. The occurrence of OA correlates with age (Arden and Newitt, 2006, A S Anderson and R F Loeser, 2010). From a health economics standpoint, this correlation is of grave concern as today's increasingly ageing demographic means the prospective number of people that may require an intervention has yet to reach its peak (P M Brooks, 2002). It is envisaged that OA will contribute a considerable burden to national GDPs and as such, there is an economic precedent for a sustained research effort into improving existing treatments and the inception of novel strategies that can effectively remedy significant cartilage injuries and arthritic joints.

A strong evidence base exists that demonstrates the role transplanted articular chondrocytes, the cells responsible for producing cartilage matrix; play in the regeneration of articular cartilage (Brittberg et al., 1994, T Minas, 2005). Cell therapies based on the concept of autologous chondrocyte implantation (ACI) are an established practice for the treatment of chondral injuries. Further value of chondrocytes lies in their ability to produce tissue engineered constructs (Yu Liu et al., 2017). Engineered cartilage may be of high value not just in the direct treatment of cartilage injury but also in providing a realistic animal model alternative for the testing of future repair strategies. Strengthening the value proposition presented by chondrocyte based cell therapies is the notion that chondrocytes may possess, to
some extent, an immune privileged status. Uncertainty remains over whether chondrocytes are immune privileged on a cellular level or rather just that the cellular immunological features are shielded by the formation of a matrix (S. Gortz and W.D Bugbee 2006., S Moskalewski et al., 2002 and A Poggi et al., 2016). In any case, the door to a new generation of allogeneic chondrocyte based treatments, be it cellular or tissue engineered, remains open and offers one such avenue for future joint care strategies.

One major problem that hampers the full potential of ACI and tissue engineered cartilage is that chondrocytes rapidly lose their phenotype during *ex vivo* expansion. To date, the *ex vivo* expansion conditions used to culture chondrocytes is heavily reliant on fetal calf serum. Fetal calf serum is a cocktail of proteins that includes Fibronectin, TGFβ1, FGF, IGF-1 in lot dependent concentrations (Zheng et al., 2006). The use of serum is at odds, due to its variable nature, with the demand for a highly characterised and controlled manufacturing process that is required for the delivery of large volumes of “quality” cells. Quality in relation to therapeutic cells is, arguably, an abstract concept. One facet of quality specific to chondrocyte expansion might be broadly summarised as the preservation of key chondrogenic genes that allow the cell to efficiently produce the cartilage matrix. Expansion of chondrocytes in serum and on tissue culture plastic leads to a dedifferentiated gene expression profile (P D Benya et al., 1982) that cannot support the production of matrix appropriate to its physiological task. Work has been done to address this by promoting chondrocyte growth and critical gene maintenance with serum-free medium formulations (Malpeli et al., 2004, P Giannoni et al., 2005). However, serum-free medium do not appear to feature routinely in experimental or clinical expansion protocols. Speculatively, this is due to the large cost associated with using a defined medium. The general approach to limit the loss
of phenotype is to either limit the expansion stage or to include a redifferentiation step in the form of pellet or scaffold culture after a period of expansion in serum-based medium.

To fully realise the potential of the role of chondrocytes in cartilage repair, be it as part of a cell-based intervention or as a tissue engineered construct, large numbers of phenotypically desirable cells that can be delivered cost-effectively is required. To achieve this elucidation of the key parameters in the nutritional regime that impact the phenotype during the *ex vivo* expansion are required and will be investigated in this thesis. Of key interest is how the time-modulated delivery of components may influence the phenotype and rate of proliferation while offering up the opportunity to utilise expensive recombinant medium components more cost-effectively.

It is envisaged that the findings presented here will contribute the creation of a time-resolved operating space that would be under the tight control of the user. In turn, this endeavour could augment established cell therapy processes that utilise viable chondrocytes, aid in the development of tissue-engineered constructs and inform novel and efficient methods of cell expansion in the future.
CHAPTER A: BACKGROUND

1.0 Cartilage

Hyaline cartilage is a tissue that covers a number of joint interface surfaces and is histologically distinct from the two other forms of cartilage – Fibrocartilage, a tough fibrous tissue found in knee joint menisci and intervertebral discs (M Benjamin and E J Evans, 1990) and elastic cartilage which contains a elastin giving it a flexible property (R W Cox and M A Peacock, 1977). Hyaline cartilage has a glassy blue appearance when fresh and is, in essence, a biphasic material - a porous structure with an interstitial fluid that gives it an elastic sponge-like property. These intrinsic properties make the tissue well adapted to handling the compressive loading and shear stresses that are felt at the joint interfaces. Furthermore, the biphasic nature of hyaline cartilage also contributes to the near frictionless movement between articulating surfaces (J Buckwalter et al., 1997). The work herein is concerned with hyaline cartilage of the articular zones and as such the term “cartilage” will refer solely to the hyaline variant unless otherwise stated. The resistance of hyaline cartilage to wear incurred in daily life is quite remarkable, especially when considering man-made biomaterials used in total arthroplasty (joint replacement) typically start to fail after ten years (D W Murry et al., 1993). This remarkable resistance profile is marred only by the tissues extremely limited capacity to regenerate from injury. This ultimately means that what starts as a minor acute cartilage injury can in some instances slowly progress towards more serious levels of degeneration which can bring with it life-changing implications (Caldwell and Wang, 2015).

Cartilage is composed mainly of water, somewhere between 60-80%, with the rest of the matrix accounted for by the extracellular matrix (ECM) - a mix of proteoglycans, link
proteins, type II collagen and other more negligible, but perhaps not insignificant components (P A Torzilli, 1985). The finer details of these components are outlined in Table 1. The dominating proteoglycan of hyaline cartilage is aggrecan, a large aggregating glycoprotein that contains within its superstructure regions of negatively charged glycosaminoglycans (GAGs) (Fig.1). These GAGs serve to attract physiological cations to the anionic regions in order to negate any net charge. In doing so a large osmotic gradient is created, allowing the movement of water into the matrix. The collagen fibres give a degree of support to the cartilage matrix, resisting the swell pressure created by the water content (Riesle et al., 1998, E Han et al., 2011). The collagen fibril network serves to keep the negative charges of the GAGs at a fixed distance apart. The absence of collagen would mean the electron-electron repulsion would alter the fixed charge density and ultimately alter the physical properties of the cartilage. The intricate organisation of the collagen fibrils differs by zones (Fig.2) and adds a depth of architectural complexity to the tissue (N Cohen et al., 1998). The exact composition as outlined in table 1 reportedly differs slightly between species, for example, unpublished data by J Rieppo and colleagues suggested bovine cartilage has a slightly lower total proteoglycan content when compared to a human.

### Table 1. General Composition of Cartilage

<table>
<thead>
<tr>
<th>Matrix Component</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water</strong></td>
<td>~60-85%</td>
</tr>
<tr>
<td><strong>Collagen</strong></td>
<td>~10-30%</td>
</tr>
<tr>
<td>II</td>
<td></td>
</tr>
<tr>
<td>I,III,VI,IX,XI</td>
<td>*** &lt; 5% cumulative</td>
</tr>
<tr>
<td><strong>Proteoglycan</strong></td>
<td>3-10%</td>
</tr>
<tr>
<td>Aggrecan</td>
<td></td>
</tr>
<tr>
<td>Versican</td>
<td></td>
</tr>
<tr>
<td>Decorin</td>
<td>*** &lt; 5% cumulative</td>
</tr>
<tr>
<td>Lumican</td>
<td></td>
</tr>
<tr>
<td>Fibromodulin</td>
<td></td>
</tr>
<tr>
<td>Biglycan</td>
<td></td>
</tr>
<tr>
<td><strong>Link Protein</strong></td>
<td>Negligible</td>
</tr>
<tr>
<td><strong>Hyaluronan (HA)</strong></td>
<td>Negligible</td>
</tr>
<tr>
<td><strong>COMP</strong></td>
<td>Negligible</td>
</tr>
</tbody>
</table>
Figure 1. The chemical structures of the two glycosaminoglycans present in the proteoglycan aggrecan: chondroitin-6-sulphate (left) and keratin sulphate (right). Represented bottom is a section of an aggrecan chain that depicts the role of other molecules such as hyaluronic acid.

Figure 2. General superstructure and organisation of cartilage. The absolute thickness of the cartilage is related to the joint of which it belongs and can vary between 1 and 6mm (D Shepherd and B Seedhom, 1999). Image taken from C V N Pullicino and J B Richardson, Basic Science for FRCS, p95)
The cartilaginous matrix is maintained by the chondrocyte. The chondrocyte is responsible for producing the components outlined in Table 1 as well as catabolic proteins that regulate normal matrix turnover (B J Rose and D L Kooyman, 2016). Although the chondrocyte is often regarded as the sole cell type present in cartilage, it should be noted that the cell fulfils different functions based on the region of cartilage it resides in. In recent years, a growing evidence base indicates that there is a distinct progenitor subpopulation present in the cartilage with more mesenchymal-like properties (G P Dowthwaite et al., 2004., R Williams et al., 2010., DD Campbell and M Pei, 2012). It is suggested that these progenitors reside in the top resting layer of cartilage (“STZ,” fig.1). Below the top superficial zone is the midzone that contains rounded proliferating chondrocytes that organise into columns. The proliferating column chondrocytes give way to the pre-hypertrophic and hypertrophic chondrocytes of the deep zone. The fully differentiated hypertrophic chondrocytes have a markedly different gene expression profile to that of resting and proliferating chondrocytes. Terminally differentiated chondrocytes create a pro bone forming environment which results in the calcified cartilage region denoted by the “tidemark” (L Yang et al. 2014). The contribution of chondrocytes from these different regions to the success of ACI interventions is not yet clear, but further research will likely aid in informing future cartilage repair therapies (C R Chu and N Friel, 2012).

The matrix environment where the chondrocyte resides is avascular and so nutritional sustenance is thought to be provided largely by diffusion from the surrounding synovial fluid. Subsequently, chondrocytes are thought accustomed to a low oxygen environment of between 0.5 and 5 % (C T Brighton and R B Heppenstall, 1978). This correlates with a relatively low base rate of metabolism. For example, complete collagen II matrix turnover in humans is thought to take approximately four hundred years which explains in part why the tissue has extremely limited capacity to recover from injury (A Maroudas, 1979). With this stated it is
perhaps unclear why chondrocyte implantation is sometimes successful in regenerating hyaline-like cartilage as the technique does not typically stimulate a significant bleed or access the mesenchymal niche of the subchondral bone which are thought to be factors in traditional wound regeneration.

2.0 Chondral Disorders

2.1 Osteoarthritis:

Osteoarthritis (OA) is a disease that afflicts the articulating joints of a range of species. It can be broadly characterised by the progressive degeneration of the hyaline cartilage that covers the joint ends combined with the general narrowing of joint spaces and the formation of small boney growths known as osteophytes (JH Kellgren and J S Lawrence, 1954). Associated with the above radiological characteristics is the manifestation and emergence of the clinical symptoms; chronic pain reduced joint mobility and an overall reduction in quality of life (J W P Michael et al., 2010). The risk factors that determine the likelihood of developing OA are not entirely clear but thought to be the result of the convergence of several factors that include genetic predisposition, lifestyle, and gait alteration (V N P Cassar and J B Richardson, 2004). The occurrence of OA generally correlates with age, although OA is not exclusive to the elderly (NICE, 2008). From a health economics standpoint, this correlation is compounded by an increasingly ageing demographic which ultimately means the prospective number of people that will require treatment has yet to peak. Current available estimates put the cost of OA in the UK at 1% of GDP (NICE,
2008) and it is envisaged that OA will contribute a considerable burden to national GDPs worldwide and as such, there is an economic precedent for a sustained research effort.

To date, there is little in the way of effective treatment for OA, with current strategies either based around palliative management or arthroplasty (joint replacement or resurfacement). The success in improving quality of life with joint replacement surgery is contradicted in part by the high cost, demand for expert professionals and the limited half-life of the implants. The lifespan of implants is a particularly troubling factor for younger patients requiring total arthroplasty as each successive revision increases the incidence of complication (K L Ong et al., 2010). These drawbacks ultimately raise questions over the sustainability of conventional OA treatment strategy as demand rises. With the acknowledgement that OA is a condition that presents without a single exclusive pathology, it is perhaps conceivable that its effective alleviation or indeed prevention may arrive in the form of a combination treatment (P Sarzi-Puttini et al., 2005). Effective treatment regimes that address pathologies relating to altered gait, muscular atrophy, bone changes, hyperplasia of the synovial membrane, morphological changes to the joint capsule and degradation of the hyaline cartilage will all likely to play a part in the restoration of normal joint function. Arguably, the role of cartilage in OA is the most scrutinised and the depth of literature in the field will aid in the development of strategies and interventions aimed at reversing localised chondral injuries.
2.2 Cartilage lesions and Osteochondritis dissecans (OCD) of the knee

Defects of the knee joint that can contribute to the development of OA, as eluded to earlier, are numerous and subject to debate. Events such as ligament tears, particularly damage to the anterior cruciate ligament and fractures of the surrounding bone, damage to the meniscal cartilage (M Englund et al., 2009) can lead to abnormal gait. Alterations to the normal pattern of movement are thought to be a possible contributor to early-stage OA (M Henriksen et al., 2010). The mechanism for this is not immediately clear, but one train of thought is that areas of cartilage experience compression and sheer from loading they have not been conditioned to. Changes to natural gait undoubtedly play a significant role in disease progression and as such comprehensive treatment packages may need to address a certain biomechanical component rather than be solely focused on treatment of the hyaline and bone surfaces.

A second recognised contributing factor is direct tissue injury to the cartilaginous surface, either acutely or through an underlying pathology. Injuries of this nature can broadly be separated into OCD or cartilage lesions with the latter being the more common. Classification of cartilage lesions and OCD are outlined in Figure 4, A and B, respectively. A cartilage lesion is an area that presents itself as abnormal upon arthroscopic investigation or MRI. OCD is a pathologically distinct occurrence where the articular cartilage and subchondral bone separate from the surface of the joint. The condition is thought to be caused by avascular necrosis of the underlying subchondral bone which in turn disrupts the articular cartilage (L Peterson et al., 2003). Both articular cartilage lesions and OCD are good candidates for cell-based therapies, and such practices already form part of adopted practices.
Fundamental to evaluating an intervention is the recognition of appropriate measurement systems that are capable of quantifying efficacy. Historically this has been accomplished using clinical examination, imaging scans and biopsy analysis. While these measurements are still of great significance, they do not always correlate with the patient's perception of the outcome. As a result, there has been a shift to patient administered reporter systems. These systems are concerned with comparing aspects such as perception of pain,
joint function and quality of life preoperatively versus postoperatively / follow up. The grading systems allow for a useful basis to establish a baseline from which interventions can be evaluated for efficacy. Advocates of such systems point out that they are less prone to potential systematic bias that can creep in if the administration is dealt with exclusively on the clinical end (M E Roos et al., 1998).

The International Cartilage Repair Society (ICRS) is a forum that brings together practising clinicians, research clinicians and research scientists who are involved in cartilage research and repair. The combined expert input to the forum has perhaps, not surprisingly, yielded numerous useful outputs. One such output is the appointment of a widely adopted evaluation system for grading defects of the knee and evaluating post-operative repair success. This is known as the ICRS cartilage injury evaluation package (Table 2) and includes both subjective patient self-assessment forms and an objective professional assessment (H Hauselmann et al., 2000). The patient administered section, however, is not universally employed and there are numerous other subjective measurement systems for assessing injury and evaluating outcome cited in the literature. The IKDC (International Knee Documentation Committee) evaluation package, the KOOS (Knee injury and Osteoarthritis Score) system, the modified Cincinnati Knee Rating System and The SF (short form)-36 are examples of perhaps the most commonly employed patient administered reported systems. The patient assessment forms tend to follow a similar formula, posing a range of questions relating to function, pain and quality of life which is then manipulated into score. Higher scores converge on normal joint function; with lower scores indicative of a greater level of symptom. The administering clinicians appear to have their preferences on which self-assessment form to employ although it is not uncommon to see a single study employ multiple subjective measures. All subjective evaluation systems have various mooted advantages and disadvantages and the chosen assessment used will likely fall to preference.
Numerous more exist and the goal is not to pragmatically debate the relative merits but rather illuminate the diversity in evaluating the efficacy of treatments in the field. There are a few studies offering insight into which objective based methods are most appropriate (K Hambly and K Griva, 2008, J Agel and R F LaPrade, 2009)

Ultimately, evaluating the intervention choice often comes down to comparing the improvement in post-operative score to the baseline score. The ICRS propose an objective surgical assessment that generates a score based on three categories (fill depth or graft survival, integration to border and macroscopic appearance). Interestingly there is a biopsy option for histological analysis but the result does not appear to contribute to the final score which ties into the earlier point made that to date, no study has been able to conclusively correlate a successful histological or radiological outcome with patient clinical outcome.

Table 2. The two parts, A and B, to the ICRS knee evaluation package

<table>
<thead>
<tr>
<th>Part A: Patient administered</th>
<th>Part B: Clinician administered</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ICRS Injury Questionnaire</td>
<td>• ICRS Knee Surgery History Registration</td>
</tr>
<tr>
<td>• The IKDC Subjective Knee Evaluation Form-2000</td>
<td>• IKDC Knee Examination Form 2000</td>
</tr>
<tr>
<td></td>
<td>• ICRS-Articular Cartilage Injury Mapping System</td>
</tr>
<tr>
<td></td>
<td>• ICRS-Articular Cartilage Injury Classification System</td>
</tr>
<tr>
<td></td>
<td>• ICRS-Osteochondritis Dissecans (OCD) Classification System</td>
</tr>
<tr>
<td></td>
<td>• ICRS-Cartilage Repair Assessment System</td>
</tr>
</tbody>
</table>
4.0 Current Standards of Care

The current standard of care for treating chondral injury is evaluated on a patient-specific basis and considers factors including the nature of the lesion (ICRS/OCD grade 1-4), size, location and any previous interventions. Established therapies can be broadly categorised into palliative, reparative, and restorative techniques.

4.1 Palliative Treatment

Debridement and Lavage

Debridement (removal of dead or damaged tissue) and lavage (wash out) are suitable for candidates who present with small lesions typically less than 2-3 cm² and complain of predominantly mechanical type symptoms such as “catching” or locking sensations. The procedure is reported to alleviate mechanical symptoms in the short term but ultimately is palliative in nature as no attempt to repair the injury is the made (K Strachan, 1992). Advocates also recommend the strategy for OA lesions as the washing out of inflammatory mediators and calcium phosphate crystals is thought to be beneficial although this remains controversial (D T Felson and J Buckwalter, 2002).
4.2 Restorative Techniques

Mosaicplasty, formally known as Osteochondral Cylinder Transplantation (OCT) is a technique that requires the harvest of either an allogeneic or autologous cartilage sample (sometimes referred to as osteochondral autograft transfer or OAT) “plug” which can then be transplanted directly into the injury site. There is an obvious limitation in using autogenic material in the sense there is a limit on the amount of material that can be harvested from the condyle margins or trochlea groove, typically 1-4cm$^2$ (L. Hangody and P. Füles, 2003). As with the majority of autograft procedures, donor site morbidity is a primary concern. Using allogeneic grafts circumnavigates this problem although more fundamental limitations such as physically accessing harvest/defect site and achieving a smooth convex finish are sustained. Allografts inevitably introduce new problems such as adventitious agents, availability of donors and potential immunogenic side effects.

The principal advantages of OCT is that the procedure can be performed in one step and achieves instant defect fill although lateral integration of graft and host matrix is reportedly rare and can lead to subsequent post-operative complications. A prospective, randomised study conducted by Bentely et al. (2002) reported that after a mean follow up time of nineteen months, the reparative practice of ACI placed 88% of patients in the good to excellent category (modified Cincinnati and Stanmore scores). In comparison, the OCT group placed 69% within the same classification. Arthroscopic evaluation revealed good to excellent repair in 82% of ACI cases as oppose to 34% after OCT and further reported 100% failure of grafts performed in the patellar region. The authors questioned the contribution OCT will make to the field of chondral defect repair in the future.
4.3 Reparative Techniques

4.3.1 Marrow Stimulating Techniques

Abrasion arthroplasty, subchondral drilling and microfracture are a series of related techniques whose restorative mode of action lies in the formation of a clot, achieved by accessing the mesenchymal niche beneath the subchondral plate (M R Steinwachs et al., 2008). Abrasion arthroplasty involves the initial debridement of the cartilage lesion followed by subsequent debridement of subchondral bone in order to achieve a bleed (L L Johnson, 2001). Subchondral drilling and microfracture are in principle the same technique – a method of breaching the subchondral bone to induce the formation of a clot. The key difference being the drilling technique, as the name suggests the use of a high-speed drill achieves the breach (M Hamanishi et al., 2013), whereas microfracture employs an arthroscopic awl (Y M Yen et al., 2008). Microfracture offers a few advantages of subchondral drilling; firstly, clinical outcome is observed to be higher than drilling. It is theorised that this is maybe due to the manual awl avoiding thermal necrosis of adjacent tissue created by the drill and the rougher surface created from the awl puncture helps facilitate clot adherence. From a practical point of view, some clinicians also find it easier to employ an arthroscopic awl. Microfracture generally has a favourable short-term outcome (K Mithoefer et al., 2009) combined with the fact the procedure is technically less demanding and relatively cheap to employ arguably gives microfracture its “gold standard” status for repair of full-thickness defects (ICRS grades 3-4) less than 3-4cm². Most products in development are compared against microfracture as a means of evaluating efficacy.
A few notable drawbacks, however, are that results histologically show that repairs are predominately fibrocartilage. The fibrocartilage repair does not have the same long-term resistance as hyaline cartilage and is thought to wear faster which draws questions over microfractures ability to affect long-term repair (A Gobbi et al., 2014., R M Frank et al., 2017). Immediately after the procedure the newly formed clot is considered extremely fragile and can be easily dislodged, to avoid this a high level of patient compliance is required postoperatively. An extrapolation of the microfracture approach termed autologous matrix-induced chondrogenesis (AMIC) sought to enhance the outcome by providing a collagen scaffold that aids clot formation and provides a structure to guide repair tissue formation (Behrens et al., 2004). This, in turn, allows larger size defects to be treated with this procedure. Short-term and mid-term results suggest an improvement in patient-reported outcome, however, the paucity of long-term data and variation in how the procedure is executed clouds overall efficacy (A Fontana., 2017, N Shaikh et al., 2017).

4.3.2 Autologous chondrocyte implantation (ACI)

Autologous chondrocyte implantation or ACI is a technique first conceived in the late 1980s, with first in man studies following in 1994 ACI is a two-step procedure that repopulates a lesion with the patient’s own ex vivo expanded chondrocytes (M Brittberg et al., 1994). The first stage of ACI involves inspection of the lesion and a cartilage biopsy taken from a non-weight bearing region of the afflicted joint. In the instance of the knee (where ACI is employed more routinely than hip or ankle), this would typically be the peripherals of the femoral condyles or the trochlea groove. The tissue explant is processed to release the chondrocytes in a GMP certified lab and the cells expanded in monolayer over a
number of weeks in the patient’s own serum. After the expansion period the cells are suspended in the patient’s serum ready for implantation in a second round of surgery. The clinician will typically prepare the lesion with an initial debridement to remove unstable tissue. To secure the transplanted chondrocytes, a periosteal flap is manufactured from an area of periosteum harvested from the patient’s proximal tibia. The flap is sutured in place and the cells injected under the patch. The post-operative care procedure is similar to that of microfracture as the repair site is fragile until cell adherence takes place.

Long-term (ten year) data is now becoming available, and the results largely ratify ACI as a viable treatment option. Two follow-up studies published 2014 both indicate satisfactory results although the authors acknowledged the procedure ultimately fails to achieve full tissue restoration and joint function (K Zaslav et al., 2009., P Niemeyer et al., 2014., S Z Nawaz et al., 2014). Transplant survival beyond ten years was assessed to be approximately 50% although the observers commented that younger patients were far more likely to have prolonged graft survival, the reasons for which are explored below. At the conception of the studies, there was little in the way of candidate screening which ultimately masks the overall potential of the procedure. For example, patients who have undergone previous marrow stimulating techniques are significantly more likely to fail an ACI procedure (T Minas et al., 2009). Incidentally, a 2004 study comparing ACI and microfracture at two year follow up reported no significant difference in outcome (R Jones et al., 2004)

There are a few practical drawbacks to ACI, an obvious limitation being the requirement for access to a GMP facility to expand the chondrocytes and secondly, the need for two separate sessions in theatre. This arguably has a sizable impact on the health economics front when compared to conceptually simpler acellular techniques like
microfracture. The national institute for health and care excellence (NICE) in the UK outlined the cost for material acquisition per patient from various sources (Table 3).

**Table 3.** Figures from a 2005 NICE Appraisal Determination for the use of autologous chondrocyte implantation for the treatment of cartilage defects in knee joints. The cited cost was adjusted for inflation based on an average inflation rate of 2.89% per year obtained from the office for national statistics composite price index.

<table>
<thead>
<tr>
<th>Source</th>
<th>Cost (2005)</th>
<th>Inflation-adjusted estimate cost for 2018</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genzyme Ltd UK</td>
<td>£4000-£5000</td>
<td>£5792-£7240</td>
</tr>
<tr>
<td>BBraun/TeTec AG</td>
<td>£4000</td>
<td>£5792</td>
</tr>
<tr>
<td>Verigen UK</td>
<td>£3200</td>
<td>£4634</td>
</tr>
<tr>
<td>Geistlich Biomaterials</td>
<td>£3500</td>
<td>£5068</td>
</tr>
<tr>
<td>Robert Jones and Agnes</td>
<td>£2000</td>
<td>£2896</td>
</tr>
<tr>
<td>Hunt Orthopaedic Hospital</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Further to the practical technicalities, there are a few commonly cited clinical complications arising from ACI. These include hypertrophic growth (most commonly associated with use of periosteum), delamination and total graft failure (P Niemeyer et al., 2008). By nature of being an autologous treatment, a biopsy of the patient’s cartilage tissue is required. Herein lay several points of notable concern. Firstly, the issue of donor site morbidity. The biopsy itself creates a new chondral injury, although there is no clear consensus that this causes a direct problem (G Matricali et al., 2010). The risk is mitigated by taking the biopsy from a non-load bearing area; however this in itself creates a paradoxical situation as the chondral population of non-weight bearing areas are thought not to be adapted to compressive and shear forces (B. Vanwanseele et al., 2002). Further to this, any underlying pathology may predispose the chondrocyte population to adoption of an undesirable phenotype. The chondrogenic potential
of the patient’s own cells is a source of potential variation that may influence outcome, which is an unavoidable part of autologous treatments. Indeed the expanded chondrocytes are not vetted for any markers of potency or quality, which remains an undefined term in itself. The biological phenomenon of senescence also limits the candidate base for ACI with the procedure generally not offered to people over fifty-five. Ultimately senescence of the chondrocyte population leads to decline in the chondrogenic potential meaning an intervention of this approach is less likely to have a successful outcome (J A Martin and J A Buckwalter, 2003). Somewhat ironically the demand for treatment may well be highest in the > fifty-five group. Interestingly though, two recent studies found the contribution of patient-specific variability and age do not correlate with the clinical outcome (O A Behery et al., 2013 and G D Smith et al., 2018). Arguably, another significant component of ACI that has the potential to affect the outcome is the conditions in which the cells are expanded (P Giannoni et al., 2005). The fine control of the expansion conditions represents an area that could yield further improvements in outcome.

4.3.3 ACI 2.0 & 3.0

Since the introduction of ACI, a host of extensions of the original procedure have been developed. The augmentations are predominantly based on the inclusion of a scaffold matrix to which the autologous chondrocytes are seeded before fitting to the lesion with a bioadhesive, typically fibrin glue. The inclusion of a matrix substrate has the practical advantage of negating the need for periosteal harvest which is beneficial for the patient, reduces surgery time and may affect outcome by bypassing hypertrophic growth associated with its use. The original extension of ACI, known as Matrix-Induced Chondrocyte Implantation, or MACI, seeds expanded chondrocytes on a porcine collagen membrane (P
Cherubino et al., 2003). The inclusion of the membrane is thought to help circumnavigate some complications encountered with periosteal ACI such as hypertrophic growth and delamination. The absolute efficacy of MACI is controversial however with studies reporting contradictory outcomes (P Niemeyer et al., 2014). Again the outcome is likely obscured by large range of variables including patient age, underlying pathology, lesion size and location, ICRS grade, expansion conditions, efficacy endpoint measure and study design. Despite the uncertainty in effectiveness a whole industry has developed offering a wide range of “ACI 3.0” options and expansion services. Products under this umbrella are sometimes referred to as MACT or “Matrix-Associated Chondrocyte Transplantation”. While the results are expected to be superior to the original ACI procedure there is a lack of evaluation studies to date.

Table 4. Overview of MACT products in various stages of development

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Description</th>
<th>Efficacy Evidence Base</th>
<th>Development Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyalograft C</td>
<td>Anika Therapeutics</td>
<td>Monolayer expanded chondrocytes seeded on a synthetic scaffold made of a hyaluronic acid derivative</td>
<td>Study conducted 2005 showed 91.5% patients showed statistically significant improvement in IKDC subjective score at short-term follow up after intervention for grade III/IV chondral lesions (average 38 months). Submitted biopsy samples were predominately hyaline-like. Further study by Brix et al. (2014) reported similar results and this concluded this MACT approach was associated with excellent outcome except patient cases deemed as “salvage”.</td>
<td>Application for centralised marketing authorisation to the EMA withdrawn as of 2013. Thought to be manufacturing related. Product future not yet known.</td>
</tr>
<tr>
<td><strong>BioCart II</strong></td>
<td>Originally Procon Biotech. Acquisition by Histogenics 2011</td>
<td>Cells expanded in monolayer with a proprietary FGF variant and then seeded onto a human fibrin and hyaluronic acid scaffold, freeze-dried to produce a porous sponge-like scaffold.</td>
<td>Results from a limited eight patient cohort for phase I study showed statistically significant increase in IKDC and Lysholm score from baseline at 6 and 12 months following intervention for grade III/IV chondral lesions. MRI finding showed between a 75% to 100% defect fill. (S E Donmayer. <em>et al.</em>, 2010)</td>
<td>Thought to be at stage II currently, however, acquisition by Histogenics may lead to development mothball in favour of perusing Neocart.</td>
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<td>---</td>
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</tr>
<tr>
<td><strong>Bioseed C</strong></td>
<td>BioTissue Technologies GmbH</td>
<td>Synthetic scaffold composed of fibrin glue and PGA, PLA, PDS copolymer seeded with autologous chondrocytes expanded in monolayer</td>
<td>Assessment at 4 year follow up of a 52 patient (Grade III/IV chondral lesions) cohort showed statically significant in t outcome scores postoperatively (IKDC) in 50 patients. (2 failures). Detailed MRI analysis at 4 years in 44 patients revealed 32 with complete defect fill, 11 with &gt;50% and 1 &lt;50% fill. MRI signal indicated normal tissue in 26 patients, small alteration in 15 and hyperintense in 3. Authors concluded Bioseed C represented a plausible treatment option for the future. (P C Kreuz. <em>et al.</em>, 2009)</td>
<td>Phase III trial currently on going</td>
</tr>
<tr>
<td><strong>Neocart</strong></td>
<td>Histogenics</td>
<td>Implant of patients own Neocartilage. The process initially expands autologous chondrocytes in monolayer before seeding onto a type 1 collagen matrix. The seeded chondrocytes are matured in a force bio reactor (which includes control over oxygen tension) for ~2</td>
<td>A randomized phase II study involving 30 patients given neocart or “next best” – microfracture) in a ratio of 2:1. Study concluded 2012 reported superior outcome in:</td>
<td>Phase III FDA trial currently on going</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• KOOS pain (at 6,12&amp;24 months)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• KOOS symptoms (6 months)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• IKDC, KOOS sports and VAS (12 &amp; 24 months)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• KOOS QoL (24 months)</td>
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</table>
weeks before further in maturation in static 3D culture. The maturation process stimulates Neocartilage formation which is subsequently transplanted. With respect to microfracture for the treatment of III/IV chondral lesions. The encouraging results are dampened by limited cohort size and variation in patient group baseline statistics. No supplementary MRI or histological analysis (D C Crawford et al., 2009)

A randomised multicentre controlled study comparing Cartipatch to Mosaicplasty for isolated focal osteochondral defects of the knee. The study concluded, at two year follow up, that Mosaicplasty returned superior IKDC scores and that functional outcome was worse in the Cartipatch group. ( A Clave et al., 2015)

<table>
<thead>
<tr>
<th>Cartipatch</th>
<th>TBF Tissue Engineering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolayer expanded chondrocytes (autologous serum) seeded on and matured on a synthetic argrose-alginate scaffold.</td>
<td></td>
</tr>
</tbody>
</table>

Product appears to have been dropped

Autologous expanded chondrocytes with attention to manufacturing variability. Product vetted for “quality.” Product / Procedure referred to as characterised chondrocyte implantation (CCI)

Clinical outcome at thirty-six months in a randomised trial vs microfracture showed CCI superior in the KOOS at endpoint with a greater number of treatment responders. The study also indicated subchondral bone reaction worsened significantly in the microfracture group ca. CCI. Interestingly the authors argued chondrocyte quality affected outcome. (D B F Saris et al., 2009)

First cell-based product registered as an ATMP with European market approval.

Table 4 gives a brief account of some of the MACT related strategies in the development pipeline. The long-term trial data of the individual approaches will undoubtedly be highly anticipated by the cartilage community so the next generation of treatments can be appropriately informed. Of equal significance is how the respective enterprises deal with
evolving regulatory requirements as well as reduce manufacturing overheads as health providers push for ever more efficient cost/benefit profiles. For example, Johnson & Johnson sought to address the drawback of a two-stage process with a point of care system in the form of a proprietary cartilage autograft system (CAIS). Briefly, the procedure involves harvest of autogenic material in a similar fashion to ACI. The harvested material is manually minced and then secured in place with a resorbable polymer scaffold and staples (J B Cole et al., 2011). Early clinical reports demonstrated safety and the phase III trial was due to conclude December 2014, however, the trial was halted after concerns relating to the cost of market entrance versus market worth were raised. It is tempting to speculate that halt was also due to the emerging technologies that avoid the need for an autograft and can access the over sixty patient demographic that is restricted in the instance of autologous approaches. One path that represents itself as a major possible augmentation is the introduction of an allogeneic cell source to MACT like products. Whilst this would introduce some new hurdles it would, like the CAIS approach, negate the need for two procedures. Moreover, an “off the shelf” product with a verified level of quality may assist in achieving a more consistent patient outcome as well as offering the chance of identifying any underlying patient pathologies that predispose an undesirable outcome. This, in turn, would allow clinicians to make more informed decisions regarding best course of patient care.
5.0 Allogeneic Solutions

5.1 Biocartilage, DeNovo NT and Revaflex

Biocartilage (Arthrex) is an “off the shelf” dehydrated allogeneic minced cartilage formulation to be used in conjunction with microfracture and platelet-rich plasma (PRP) (G D Abrams et al., 2013). There is evidence to suggest PRP has mesenchymal chemoattractant and mitogenic properties which encourage migration of the multipotent stem cells from the subchondral bone to the defect site (E Rubio-Azpeitia and I Andia, 2014). The minced allogeneic cartilage offers a pseudo-scaffold to aid in clot stabilisation and subsequent chondrogenesis. Clinical data is as of yet unavailable but is expected to be superior to microfracture. Two other products in the development pipeline of note are DeNovo Natural Tissue (NT) and Revaflex, formally DeNovo Engineered Tissue (ISTO / Zimmer). These products stand out for a number of reasons, firstly the allogeneic sources are viable and secondly, the products use “juvenile” (classified as from a donor in the ≤13 years) chondrocytes. DeNovo NT is a minced allogeneic cartilage product similar to in concept to CAIS and the established OAT procedures. The product is shipped in blister products containing nutrient medium. DeNovo NT uses juvenile chondrocytes as opposed to mature on the reported basis of the significantly increased metabolic features such as collagen II and GAG expression (D H Adkisson et al., 2010). To date, there are only a few studies evaluating the short-term efficacy of DeNovo NT (J A Buckwalter et al., 2014., Y H Stevens et al., 2014) and none have published long-term follow-ups. Further, no comparator studies to microfracture are currently available, so the efficacy of the treatment remains as of yet, unknown. Revaflex (formerly DeNovo ET) is a living tissue engineered construct that drives cultured juvenile chondrocytes to form a Neocartilage matrix which is subsequently
implanted into patients. Revaflex is thought to be currently in the middle of a phase III trial after phase II showed statistically significant outcomes and no immunological response (J D Harris et al., 2013)

6.0 The Immunogenicity of Chondrocytes

An important question that arises from the emergence of commercialised allogeneic products is the immunogenic profile of the viable chondrocytes. The long-established procedure of allogeneic osteochondral grafting leads to an intuitive belief that chondrocytes are immune privileged to an extent. A body of evidence exists that demonstrates that chondrocytes can and do express both MHC I and MHC II antigens and do provoke T-Cell proliferation (C M Revell and K Athanasiou., 2009). Additionally in vivo immunological responses were observed in rabbits transplanted with allogeneic chondrocytes, which ultimately lead to graft rejection (S Moskalewski et al., 2002). These observations are not consistent with adopted practices of allogeneic cartilage grafts which have no significant incidence of immune rejection. This lead to the generally accepted hypothesis that the avascular nature of cartilage tissue and the isolated pericellular environment of the chondrocytes probably play a significant role in shielding immunological features from the host immunocompetent cells. The established paradigm is one of evasion rather than “invisibility”, however, a growing evidence base poses to re-ignite the debate. A contradictory study involving HACs have shown no presence of MHCII, CD80 and 86 antigens by flow cytometry and subsequently failed to stimulate T-cell proliferation in in vitro models (D J Huey et al., 2012). Further to this, a 2010 study on juvenile HACs showed
they actively suppressed T-cell proliferation in a contact-dependent fashion (D H Adkisson et al. 2010). The literature appears divided on whether chondrocytes or their tissue engineered products are immunogenic or not and Table 5 illustrates the division.

**Table 5.** Overview of outcomes from studies addressing the immunogenicity question

<table>
<thead>
<tr>
<th>Isolated or Matrix</th>
<th>Species</th>
<th>Immune Reaction?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bound Chondrocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated</td>
<td>Rabbit</td>
<td>Yes</td>
<td>S Moskalewski et al, 2002</td>
</tr>
<tr>
<td>Both</td>
<td>Rabbit</td>
<td>No</td>
<td>PJ Chesterman et al, 1968</td>
</tr>
<tr>
<td><strong>Matrix Bound</strong></td>
<td>Rabbit</td>
<td>Mixed</td>
<td>B Arzi et al, 2015</td>
</tr>
<tr>
<td>Isolated</td>
<td>Human</td>
<td>No</td>
<td>P Jobanputra et al, 1992</td>
</tr>
<tr>
<td>Isolated</td>
<td>Human</td>
<td>No</td>
<td>DH Adkisson et al, 2010</td>
</tr>
<tr>
<td>Isolated</td>
<td>Human into Pig</td>
<td>Yes</td>
<td>Niemietz et al, 2014</td>
</tr>
<tr>
<td>Isolated</td>
<td>Rat</td>
<td>Yes</td>
<td>A Romaniuk et al, 1995</td>
</tr>
</tbody>
</table>
The exact nature by which isolated chondrocytes and matrix bound chondrocytes sometimes achieve their apparent immune privileged status is not fully agreed upon but seems to be the result of a combination of factors. Factors including, heterogeneity in chondrocyte immune profiles, dense ECM shielding of chondrocyte immune features and generally low T-cell infiltration into the ECM and synovial fluid. Further to this, a 2015 study concluded that the survival of a tissue engineered construct in rabbits was a function of not only the donor source but also the lesion location. Xenogeneic material was strongly rejected at all implant sites however allogenic material implanted survived in the trochlea but elicited a notable immune response in the patella.(B Arzi et al, 2015). The authors hypothesised the difference could be due to proximity of the implant site to the synovium. This discovery may explain the earlier Moskalewski study, which was also performed with rabbit recipients. Unfortunately, though the earlier study used free chondrocytes and not construct bound chondrocytes which makes direct comparison difficult. The question of lesion site adds an additional dimension of complexity to the immunological conundrum. Ultimately, the most important question that arises from the discussion takes the form of ‘is any detectable sign of immune response clinically relevant?’ The current body of evidence cannot definitely answer such a question and any answer may be dependent on multiple conditions been met.
7.0 Cell Sources for ACI or tissue engineered constructs

Treatment technology using viable cells can broadly be divided into four categories; Autologous, allogenic, xenogeneic and syngeneic. Autologous and syngeneic (genetically identical donor) cells, as already been touched upon, are patient derived. The primary advantage of autologous cells is that the immunological question of host-graft rejection is side stepped (Z Chen et al, 2008). From an industry perspective, the use of autologous cells is mixed. Regulatory restrictions are more relaxed surrounding the use of autologous cells while they remain classed as “minimally manipulated” and so the route to early clinical trials is less obstructive. This is reflected by the general observation that the literature reports a far greater proportion of trials using autologous cells. On the other hand, there are a few drawbacks to autologous cell therapies from a commercial perspective. Clinicians can prepare autologous cells on site without the need for commercial support. The nature of autologous cells means that any commercial outfit will face an almost insurmountable challenge of intimately characterising cell quality and understanding failure modes. The most notable disadvantage of autologous cells is the limited amount of material that can be harvested which can be problematic in the instance where a lot of tissue or cells are required in order to affect a treatment or the cells are required in a time critical scenario.

Allogenic, from a different individual but same species or Xenogeneic, different species are an attractive option due to the availability of material. The allure of such cell sources is increased by the notion that a “universal” cell line could be established to provide a platform for a more quality assured product. A scaled up or scaled out allogenic-based product more readily lends itself to economically efficient quality testing due to the fact the testing could be done on large batches. Additionally, and assuming the end product lends
itself to storage, the possibility of stock piling remains open which may be advantageous for products intended for medical emergencies. The major roadblock for allogenic and xenogeneic-based treatments is the immunological question. It is accepted that introducing immunologically incompatible material into the body will result in host-graft rejection which means allogenic cell therapies will require strategies to avoid this such as HLA matching, host immune suppression or immune evasion.

Outside of the direct question of allogenic or autologous the important consideration of the source tissue of the cell and the type of cell. Chondrocytes could be derived directly from cartilage such as the case with autologous chondrocyte implantation however this is not the only option. Chondrocyte progenitors can be harvested and insolated from the full thickness population present in hyaline cartilage (C Archer et al., 2004). Chondrocytes or chondrocyte progenitors can be obtained from the differentiation of mesenchymal, embryonic or induced pluripotent stem cells (A Cheng et al., 2014 and RM Guzzo et al., 2014). To add the complexity mesenchymal stem cells can be isolated from a number of different tissue sources such as bone marrow, dental pulp and adipose (P Stanko et al., 2014). In spite of the variety of potential sources on offer, an argument, based on intuition would say that the best source for a producing chondrocytes capable of supporting a permanent cartilage matrix would be one derived from the in vivo niche. To this end, chondroprogenitor isolated from full thickness cartilage are an attractive candidate for manufacturing studies that would circumnavigate the need for additional, potentially expensive, differentiation steps necessary when starting with non-committed stem cells.
7.1 Culture Conditions and Explant Procedure

Chondrocytes expanded for both clinical and research application are typically cultured using serum. The former typically employs autologous serum obtained from the patient’s blood, whereas the latter uses commercially available animal serum. Chondrocytes are an anchorage-dependent cell line and are typically expanded on tissue culture plastic in the form of T-flasks or carrier beads. A commonly cited problem with the culture of chondrocytes is the rapid loss of the in vivo phenotype which can be characterised by the loss of transcript expression of the master chondrogenic gene SOX-9 and the SOX-9 support genes SOX-6 and 5, this, in turn, leads to the loss of cartilage-specific markers. SOX-9 is a transcription factor expressed in differentiated chondrocytes but not in hypertrophic chondrocytes (Zhao et al. 1997). Studies have determined that it plays a central role in activating downstream cartilage-specific markers Col2a1, Col1a2, and aggrecan (Bi et al. 1999). SOX-9 is also thought to play a role in modulating the rate at which proliferating column chondrocytes convert to hypertrophic chondrocytes by mediating the function of parathyroid hormone-related protein (PTHrP) (W Huang et al. 2001). PTHrP forms part of a negative feedback loop with Indian hedgehog (Ihh) (H M Kronenberg et al. 2001., H Akiyama et al., 2002). Indian hedgehog protein is an activator of PTHrP. Activated PTHrP, in turn, is thought to prevent the proliferating chondrocytes which express Ihh from progressing to hypertrophic chondrocytes directly. In addition, SOX-9 is thought to be a target of PTHrP, which results in enhanced transcript expression. This intricate cascade is represented in the diagram in figure 5.
How exactly SOX-9 contributes to maintaining the stability of the proliferating and pre-hypertrophic chondrocytes is unclear but investigators observed that SOX-9 knockout mice exhibit enlarged prehypertrophic zones. The loss of the PTHrP-Ihh feedback loop promotes the emergence of hypertrophic chondrocytes, and the emergence of hypertrophy markers; collagen X expression, matrix metalloprotease-13, alkaline phosphatase and vascular endothelial growth factor (M B Goldring et al., 2006). SOX-6 and SOX-5 are co-expressed with SOX-9 in differentiated chondrocytes and work cooperatively to activate the Col2a1 gene. It was demonstrated that SOX-6 and 5 were required to achieve collagen expression levels consistent with that of mature cartilage (Smits et al., 2001).

**Figure 5.** Diagram showing the PTHrP-IHH negative feedback loop taken from H M Kronenberg (2003). (1) PTHrP is secreted from chondroblasts and chondrocytes and acts on the proliferating column chondrocytes to sustain proliferation and delay IHH production. (2) IHH is produced as the PTHrP signal diminishes. (3) IHH production stimulates production of more PTHrP at the top of the proliferating chondrocytes. (4) IHH further promotes osteogenesis by directing perichondral mesenchymal cells down the osteoblast lineage.
Loss of SOX signalling in chondrocytes leads to dedifferentiation and is thought to be instigated, at least in part, by changes to the cytoskeleton that are the result of culturing on tissue culture plastic (A woods et al., 2005). Dedifferentiation of chondrocytes can be reversed, to an extent, by employing “three-dimensional” culture methods such as pellet or incorporation of cells into gels (P.D. Benya, J.D. Shaffer, 1982). It would be advantageous, however, purely from an economic standpoint, to avoid the need for such methods by preserving the phenotype during the expansion.

7.2 Chondrocyte Culture Survey

In order to gauge the degree of variability between culture practices typically employed and to inform the proposed experimental work a brief exploration of explant and expansion protocols described in the literature was undertaken.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Cell Source</th>
<th>Harvest Method</th>
<th>Expansion Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Susante, J. L. et al. Culture of chondrocytes in alginate and collagen carrier gels. <em>Acta Orthop. Scand.</em> <strong>66</strong>, 549–56 (1995).</td>
<td>Bovine Metacarpophalangeal Joints</td>
<td>1) 2h incubation in RPMI DM Culture medium, pyruvate (1mM), gentamycin (1.2%) and Pronase E (0.2%)&lt;br&gt;2) Overnight incubation with collagenase B (0.1%), culture medium. 37°C. Air (95%), CO₂ (5%).&lt;br&gt;3) Cells washed 3 times by centrifugation and resuspended in RPMI DM (30mL)</td>
<td>Collagen Gel&lt;br&gt;Cells Dispersed in:&lt;br&gt;• 7 volumes of Collagen solution (8mg /ml in acetic acid (0.05%) UV sterilised&lt;br&gt;• 2 volumes of 3xconcentrated DMEM&lt;br&gt;• 1 volume Hepes (0.2M) to a concentration of 2 x 106 cells / mL&lt;br&gt;• 24 well plates precoated with 0.3mL gel and slabs of DMB on the bottom were prepared&lt;br&gt;• Samples containing Chondrocytes placed on top and allowed to gel at 37°C&lt;br&gt;• Gels overlaid with RPMI DM (2mL) w/ 10% FCS.&lt;br&gt;• Medium Changed every other day&lt;br&gt;Alginate Gel&lt;br&gt;• Low Viscosity Alginate dissolved in physiological saline and autoclaved for 20m.&lt;br&gt;• Pelleted Chondrocytes suspended in the alginate solution and sampled as for collagen.&lt;br&gt;• Gelled by addition of Ca²⁺&lt;br&gt;• Gel washed 4 times with saline.&lt;br&gt;• Addition of culture medium (DME / Ham’s F12 w/ 10% FCS).</td>
</tr>
<tr>
<td>Flannery, C. R., Little, C. B., Caterson, B. &amp; Hughes, C. E. Effects of culture conditions and exposure to catabolic stimulators on the expression (MMPs) and disintegrin metalloproteinases</td>
<td>• Human Articular Chondrocytes (68-79-year-old patients suffering advanced OA of the knee)&lt;br&gt;• Bovine from Metacarpophalangeal Joints (1-2 week old calves)</td>
<td>1) 0.1% Pronase in DMEM containing 5% FBS for 1 h at 37°C with agitation.&lt;br&gt;2) 0.04% collagenase in DMEM w/ 5% FBS overnight 378C with</td>
<td>Monolayer and Agarose (same conditions):&lt;br&gt;• Seed density : 1x10⁶ cm²&lt;br&gt;• DMEM&lt;br&gt;• 10ng/ml IL-1β (recombinant) or 1mM trans-Retinoic Acid&lt;br&gt;• Maintained for 4 days</td>
</tr>
<tr>
<td>(ADAMs) by articular cartilage chondrocytes. <em>Matrix Biol.</em> <strong>18</strong>, 225–37 (1999).</td>
<td>Rabbit Articular Chondrocytes</td>
<td>Alginate Gel Culture:</td>
<td></td>
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<tr>
<td>• Porcine from Metacarpophalangeal Joints (3-6-month-old)</td>
<td>agitation. Cells were filtered washed and counted.</td>
<td>• One mL aliquots of cell suspension plated on 35mm Petri dishes and gelled by addition of 2mL of CaCl$_2$ (50mM), NaCl (0.1mM), (10mM) Hapes (10mM). After gelation excess, CaCl$_2$ was removed by washing with an excess of KRH solution</td>
<td></td>
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<tr>
<td></td>
<td>2) Minced and digested at 37°C with collagenase (125 U ml$^{-1}$) for 3-5h under constant oscillation in DMEM supplemented with 2% FCS, glutamine (2mM), Penicillin (500 U ml$^{-1}$), Streptomycin (500 µg ml$^{-1}$) and Hapes-NaOH (10mM) – pH 7.4</td>
<td>• Media: DMEM supplemented with FCS (10% v/v), penicillin (500 U ml$^{-1}$), streptomycin (500 µg ml$^{-1}$), Glutamine (2mM) and ascorbate (50 µg ml$^{-1}$)</td>
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<tr>
<td></td>
<td>3) Solution was filtered and diluted with KRH (Krebs-Ringers Henseleit) solution (NaCl (110mM), KCl (10mM), MgCl$_2$ (1mM), CaCl$_2$ (1mM), Hapes (30mM))</td>
<td>• Cells cultured for one week at 37°C at 5% CO$_2$ with medium change every two days.</td>
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<td></td>
<td>4) Cells pelleted by centrifugation at 600xg for 10m</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>De Bari, C., Dell’Accio, F. &amp; Luyten, F. P.</th>
<th>Mix of cadaveric (12 hours post-mortem) and live biopsy (knee replacement surgery) periosteum-derived cells (mean age 57 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jakob, M. <em>et al.</em> Specific growth factors during the expansion</td>
<td>Human Articular Chondrocytes from hip or</td>
</tr>
</tbody>
</table>
| Samples incubated at 37°C in type II collagenase (0.15%) and resuspended in DMEM | 1) Samples rinsed twice with balanced salt solution and supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), amphotericin (0.25 mg/ml)  
2) Samples minced and digested with collagenase (0.2%) in high glucose DMEM w/ FBS (10%)  
3) Overnight incubation at 37°C then collected by centrifugation, twice washed and resuspended in high glucose DBEM w/ FBS (10%) and antibiotics.  
4) Plated in T25 culture flask and allowed to attach for 4 days  
5) Non-adherent cells removed by medium change. | Monolayer Culture: |
| | • Growth medium (implies the same mixture as before) at 37°C and 5% CO2  
• Medium change every 3 days  
• After 10-20 days of primary culture (confluence) the cells were washed with PBS and harvested with trypsin-EDTA (0.25% trypsin, 1mM EDTA) and repeated at 1:4 dilution.  
• Cell passages were continued in the same way (1:4 dilution) every 6-12 days (at confluence) | Growth Factor Treatment: |
| | • Recombinant BMP-2, BMP-4, BMP-7, GDF-5, CDMP-1 were dissolved in 45% acetonitrile and 0.1% TFA and added to the culture medium at final concentrations of 100-300 ng/mL  
Recombinant TGF-β1 was dissolved in HCl (4mM), BSA (1mg/mL) and added at a final concentration of 10ng/ml | Growth Factor Concentrations: |
| Initial Monolayer Expansion: | • Cells plated in flasks at approximately $10^4$ cells/cm² and cultured in an incubator at 37°C and 5% CO₂  
• At approximately 10 days (sub-confluency) first passage cells were detached using trypsin (0.25%) and EDTA (1mM)  
• After 10 days P2 cells were detached in the same manner and cultured in pellets with the addition of various growth factors to the control medium |

<table>
<thead>
<tr>
<th>Kolettas, E., Muir, H. I., Barrett, J. C. &amp; Hardingham, T. E. Chondrocyte phenotype and cell survival are regulated by culture conditions and by specific cytokines through the expression of Sox-9 transcription</th>
<th>ankle joint (mean age 57.5) of 4 patients not presenting with joint disease following joint replacement (femoral neck fracture) or foot amputation (tumour resection)</th>
<th>containing FBS (10%, d-glucose (4.5 mg/mL), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), HEPES buffer (100 mM), penicillin (100 U/mL), streptomycin (µg/mL) and L-glutamine (0.29 mg/mL)</th>
</tr>
</thead>
</table>
| DES4 supB+ and 10W sup B+ clone 1. (Carcinogen-immortalized, non-tumorigenic Syrian hamster embryonic chondrocyte-like cells) | N/A | • FGF-2 (5 ng/mL)  
• EGF (10 ng/mL)  
• PDGFbb (1 ng/mL)  
• TGF-β (1 ng/mL)  
• All used with control medium (left) to study population doublings during P1  

Pellet Culture:

- Both serum free (SFM) and serum supplemented formulations employed.  
- SFM: DMEM with ITS+1 (sigma), ascorbic acid 2-phosphate (0.1 mM) and HSA (1.25 mg/mL)  
- Serum supplemented medium was control medium (left) with insulin (10 mg/mL) and ascorbic acid 2-phosphate (0.1 mM) (concentration was of insulin was to substitute requirement for IGF-I (Yaeger et al., 1997))  
- Aliquots of 5x10^5 in medium (0.5 mL) were centrifuged at 7500 rpm (15 s) in polypropylene conical tubes to form spherical pellets.  
- Pellets were placed onto a 3D orbital shaker at 30 rpm in an incubator humidified at 37°C under 5% CO₂  
- Pellets were cultured in 4 different mediums (0.5 mL) : Serum supplemented, Serum-free(SF), SF with TGF-β (10 ng/mL) and SF with TGF-β and dexamethasone (10^-7 M)  

| Kolettas, E., Muir, H. I., Barrett, J. C. & Hardingham, T. E. Chondrocyte phenotype and cell survival are regulated by culture conditions and by specific cytokines through the expression of Sox-9 transcription | N/A | • Cells were cultured as monolayers in MEM with FCS (10%), non-essential amino acids (1% v/v), L-glutamine (1.4 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C under 5% CO₂  
• Early passage cells were split 1:16 and expanded in the above medium for 3 days (50-60% confluent) or 4-5 days for fully confluent.  
• The medium was changed either with the same control medium or a serum-free growth medium with or without human recombinant IL-1α (5 ng/mL) or IGF-1 (50 ng/mL), or both together.  
• A selection of early passage cells grown either in the default or serum-free medium were treated with 5-azacytidine (4.10 µM) |

<table>
<thead>
<tr>
<th>Human Ear Cartilage from three children (5-10yrs) during ear correction surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) The perichondrium was dissected from the cartilage. The cartilage was sliced into small pieces and washed several times in physiological saline.</td>
</tr>
<tr>
<td>2) Two-stage digestion protocol for cell release: Cartilage was pre-incubated with pronase E (2 mg/mL) for 2h followed by overnight digestion with collagenase B. Cells were isolated by filtration (100μm) to remove undigested cartilage.</td>
</tr>
<tr>
<td>3) Cell viability was tested using trypan blue</td>
</tr>
</tbody>
</table>

Monolayer Expansion:

- Four different types of media were prepared: DMEM with FCS (10%), DMEM with ITS\(^+\) (1:100), DMEM with ITS\(^+\) and recombinant FGF\(_2\) at two concentrations (5ng/mL and 100ng/mL)
- All growth mediums were supplemented with gentamycin (50 mg/mL) and fungizone (1.5 mg/mL)
- Cells seeded at a density 4x10\(^4\)
- Chondrocytes trypsinized at subconfluency (trypsin-EDTA)
- Start of each new passage serum-free conditions were supplemented with FCS(10%) for a day to promote adherence
- Medium was changed every other day
- Cells expanded for four passages

Redifferentiation Culture:

- Chondrocytes from the four different conditions were encapsulated individually.
- Cells were suspended in alginate (1.2%) in saline at a density of 4.3x10\(^6\) cells/mL
- The suspension was dropped into a CaCl\(_2\) (102mM) solution via needle to create the gelled beads
- After 10m the beads were washed three times with physiological saline
- DMEM was supplemented with ITS\(^+\), IGF-1(10 ng/mL), TGFβ2 (10 ng/mL) and hydrocortisone (0.1 mg/ML). L-Ascorbic was added (25 mg/mL)
- Described previously to induce dedifferentiation
Malda, J. *et al.*

### Control
- Control was set up in the form of bead culture with DMEM w/ FCS (10%).
- Beads were cultured for 21 days in a 24 well plate.
- Each well contained 10 beads and 50 mL of medium.
- Medium was changed every other day.

### Microcarrier Selection, Seeding, and Culture
- Suitability of several commercially available microcarriers decided by initial cell attachment (3h).
- Cytodex 1 microcarriers were chosen.
- Microcarriers steam sterilised and washed with PBS twice, then with culture medium (37°C).
- Expansion was achieved by transfer of microcarriers to a Sigmacote-treated 250mL spinner flask with a working volume of 100mL, end concentration of 3mg of Cytodex per mL.
- Microcarriers seeded (3500 cells/cm²) according to an intermittent stirring regime (30 min at 0 rpm, 1 min at 20 rpm for 3h).
- After seeding the stir rate was increased to 30 rpm.
- Culture was maintained in a humidified 5% CO₂ incubator at 37°C.

### T-flask Culture
- Seeded at a density of 3500 cells/cm² in T 175 Flasks and maintained in a humidified 5% CO₂ at 37°C.
- After 8-9 cells reached confluence and were harvested.
- Harvest was achieved by separation of cells from microcarriers by an initial 1h collagenase treatment followed by filtration through a Falcon filter (100-mm pore size).
- Microcarriers were rinsed with PBS to remove trace collagenase.

| Bovine Chondrocytes from the femoral condyle (6 months old calves) | 1. Tissue dissected into 1-mm cubes and digested overnight with type II collagenase (0.15%).
2. Cells washed three times with PBS then suspended in HEPES buffered DMEM w/ FCS (10%), ascorbic acid 2-phosphate (0.2mM), non-essential amino acids (0.1mM), proline (0.4mM), penicillin (100 units/mL) and streptomycin (100 mg/mL). |
| --- | --- |
Cells were washed three times with PBS then centrifuged for 10 min at 300 x g
- Cells were resuspended in culture medium ready for a viable cell count (trypan blue)

**Pellet Culture**

- Resuspended cells (500, 000 per tube) centrifuged with culture medium (1.5mL) for 2m at 300 x g.
- The resulting pellets were cultured statically at standard CO$_2$/Temp. Media was changed every 2-3 days.

**Monolayer Culture**

- Cells seeded in flasks at a density of 1x10$^4$ cell/cm$^2$
- Cells cultured either with control medium (left) or with the addition of TGF-β1 (1ng/mL), FGF-2 (5 ng/mL), PDGF-BB (10 ng/mL) in a humidified 37°C / 5 % CO$_2$ atmosphere. The growth factor combination was based on previous reports that their presence promotes proliferation and redifferentiation.
- After approximately ten days cells were 80% confluent
- Cells were passaged and rinsed with PBS and detached using trypsin-EDTA (0.05%-0.53mM)
- Cells reseeded at 5x10$^3$ cells/cm$^2$
- At 80% confluence (Approx. 1 week) the second passage cells (equivalent to a total of 4.6 ± 0.9 and 8.1 ± 1, for control and GF enhanced respectively) were introduced into pellet culture.

<table>
<thead>
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<tbody>
<tr>
<td>Cadaveric human chondrocytes taken 24h post-mortem from 69 patients with no history of joint disorders (age range 20-91).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 1) Cells isolated with type II collagenase (0.15%, 10mL/g tissue) for 22h.  
2) Cells were suspended in DMEM v/ FCS (10%) and penicillin (100 U/mL) / streptomycin (100 µg/mL) |
<table>
<thead>
<tr>
<th>Pellet Culture</th>
<th>Monolayer expansion:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Pellet culture used a serum free medium consisting of DMEM supplemented with ITS(^{+1}), ascorbic acid 2-phosphate (0.1 mM), human serum albumin (1.25 mg/mL), dexamethasone (10(^{-7}) M) and TGF-β1 (10 ng/mL)</td>
<td>• Cells were seeded at a density of 50,000 cells/cm(^2) in T75 flasks in culture medium</td>
</tr>
<tr>
<td>• Aliquots of 5x10(^5) cells / 0.5 ml were centrifuged at 250xg for 5 m in propylene conical tubes</td>
<td>• Culture medium: DMEM/F-12, FCS (10%), amphotericin B (2.5 mg/ml), penicillin (100U/mL), streptomycin (100mg/mL), bovine insulin (1mg/mL) and ascorbic acid (50 mg/mL)</td>
</tr>
<tr>
<td>• The pellets were placed onto a 3D orbital shaker at 30rpm</td>
<td>• Cells cultured at 37°C in humidified 5% CO(_2) atmosphere for 24h</td>
</tr>
<tr>
<td>• Pellets were cultured for two weeks with media changes twice weekly before analysis.</td>
<td>• Cells were checked for adherence and non-adherent cells were removed</td>
</tr>
</tbody>
</table>

**Cadaveric human articular cartilage** (18 subjects between 32 and 89, harvested within 24h post mortem)

1. Cartilage specimens kept in transport medium (DMEM / Nutrient mixture F12, amphotericin B (2.5 mg/ml), penicillin (500U/mL) and streptomycin (500mg/mL)) until processing (within 24h of biopsy)
2. Specimens were diced in PBS
3. Diced tissue was incubated with pronase E (10 ml/g of wet tissue) in a shaker water bath for 60m at 37.8°C
4. Pronase was removed and the cartilage specimens were washed with PBS
5. Collagenase P in digestion medium (10ml/g wet tissue) (Digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; digestion medium; 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DMEM/F-12, bovine insulin (1mg/mL) and ascorbic acid (50 mg/mL) was added and left overnight at 37.8°C in a shaking water bath
6. The suspension was filtered through a nylon strainer (100mM)
7. Cells were centrifuged then washed twice with PBS before counting then either cryopreservation

** Note, the authors felt this method was superior to isolation procedures using just collagenase in terms of both yield and capacity for attachment. **

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Giannoni, P. *et al.*

Autologous chondrocyte implantation (ACI) for aged patients: development of the proper cell expansion conditions for possible therapeutic applications.


Human articular chondrocytes from five patients (69-75 years) undergoing knee arthroplasty

Controls were taken from three young patients (27-35) undergoing reparative surgery

1) Cartilage was cleared of connective tissue / subchondral bone
2) Specimens were minced into small fragments and rinsed with PBS (pH 7.2)
3) Enzymatic digestion achieved with hyaluronidase (1mg/mL), collagenase I (400 U/mL), collagenase II (1000 U/mL) and 0.25% trypsin in PBS at 37°C
4) Cells were pooled and counted

- The culture dishes used for expansion were pre-coated with 10% FCS in Coon’s modified Ham’s – F12. This was to allow attachment of the serum fibronectin to the plastic.
- After 24h the dishes were washed three times with PBS to remove all FCS
- The primary chondrocytes were kept in FCS containing medium for 48 and then detached and washed with PBS ready for culture
- HACs from each donor were divided into equivalent aliquots so that one could be grown in the serum-free medium and the other in serum-containing medium.
- The SF medium was Coon’s modified Ham’s F-12 supplemented with
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>250 mM</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>4.5 mM</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>13 mM</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>10 nM</td>
</tr>
<tr>
<td>N-acetylcyesteine</td>
<td>50 mM</td>
</tr>
<tr>
<td>Apo-Transferrin</td>
<td>25 mg/mL</td>
</tr>
<tr>
<td>Holo-Transferrin</td>
<td>25 mg/mL</td>
</tr>
<tr>
<td>Sodium Selenite</td>
<td>30 nM</td>
</tr>
<tr>
<td>Sodium Pantothenate</td>
<td>17 mM</td>
</tr>
<tr>
<td>Biotin</td>
<td>33 mM</td>
</tr>
<tr>
<td>Insulin</td>
<td>10 mg/mL</td>
</tr>
<tr>
<td>EGF</td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>FGF-2</td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>PDGF</td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>Human Albumin</td>
<td>1%</td>
</tr>
</tbody>
</table>

- Medium was changed three times a week
- Cell passaging and counting was performed at sub confluence
- After five population doublings, cells were trypsinized then used either for analysis or to test chondrogenic potential
- Alternative procedure was to culture cells with FCS for five population doublings
- The effects of TGF-β1 and trans-Retinoic acid were tested by addition to some batches as well

General

- Cells were seeded at a density of $4 \times 10^4$ cells/cm$^2$ in plastic vessels
Duval, E. *et al.*

Hypoxia-inducible factor 1alpha inhibits the fibroblast-like markers type I and type III collagen during hypoxia-induced chondrocyte redifferentiation: hypoxia not only induces type II collagen and aggrecan, but it also inhibits type I and type III collagen. *Arthritis Rheum.* **60**, 3038–48 (2009)

<table>
<thead>
<tr>
<th>Hypoxia Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Culture medium consisted of DMEM w/ FCS (10%), penicillin (100 IU/mL), streptomycin (100g/mL) and fungizone (0.25 g/mL) in an atmosphere of 5% CO₂</td>
</tr>
<tr>
<td>- Medium was changed every two weeks</td>
</tr>
<tr>
<td>- At confluence, cells were enzymatically harvested with trypsin-EDTA (0.25% / 1 mM) counted and seeded again at the same density.</td>
</tr>
</tbody>
</table>

**Human articular chondrocyte from femoral heads of patients undergoing joint arthroplasty (age range 37-81).** Samples healthy in appearance by macroscopic analysis were used.

1. Samples were cut into small pieces and ready for a sequential digestion
2. Samples were initially incubated at 37°C for 45m with protease XIV (2mg/ml) followed by an overnight digestion with collagenase I (1mg/mL)
3. The cell suspension was filtered through a nylon mesh membrane (70µm) before centrifugation

**Alginate Bead Redifferentiation Studies:**

- Cells were aliquoted, centrifuged and suspended in sodium alginate at a density of 10⁷ cells/mL.
- The beads were formed by slowly dispensing droplets of the solution-suspension through a needle (.22 gauge) into a solution of CaCl₂ (100mM)
- The CaCl₂ was removed and the beads washed with NaCl (0.15M) then washed with DMEM w/ FCS (10%) before culturing with the same medium.
| Adkisson, H. D. *et al.* | Cadaveric human articular chondrocytes from 65 different donors (19 from ranges 4-79 and 46 from ages spanning birth to 13, classed as juvenile) | 1) knees en bloc” were received on ice and processed in accordance with current good tissue practices  
2) Cartilage was taken from the proximal femur and the distal tibia  
3) The cartilage was minced and cells released by initial treatment with pronase (0.2%) followed by overnight digestion with a mixture of collagenase(0.07%) and hyaluronidase (0.04%) in the presence of a proprietary chemically defined serum-free medium HL-1 (Lonza) | Neocartilage  
- Non expanded chondrocytes were seeded in a un specified vessel at a density between 0.5 – 1x10⁶ cells/cm²  
- HL-1 medium was again used but sometimes substituted for an alternative defined medium that was thought to display similar growth characteristics to the HL-1  
- Cells were cultured in an incubator at 37°C in a humidified environment (5% CO₂) and supplemented with ascorbate (50 mg/ml) after day 3  
- Medium exchange was carried out every 3-4 days  
- The Neocartilage was harvested between days 44 and 63 of culture for characterisation  

Population growth and differentiation culture  
- Note the chondrocyte isolation method was slightly different than described earlier: The sequential digestion was absent hyaluronidase and the medium used was DMEM v/ 10 % FCS.  
- Cells were plated in monolayer culture and grown for 5 to 10 days before passaging (~80% confluence) with trypsin  
- Replicates at 1:3 split  
- Cells cultured for up to 104 days  
- Population doublings were tracked through time based on cell counts at each passage. | 1) Cartilage samples were cut into small pieces and transferred to a Petri dish | Monolayer Culture: |
2) Sliced cartilage were maintained overnight in Ham’s-F12: DMEM w/FBS, ascorbic (100mg/mL), penicillin (100 U/ml) and streptomycin (100mg/ml) at 37°C in 5% CO₂ atmosphere. The overnight cool off was to ensure sterility of specimens before digestion

3) The exact duration of the enzymatic sequence was dependant on the size of the cartilage sample

4) The cartilage was first washed in buffered PBS containing antibiotics then incubated with trypsin (0.25% w/v) at 37°C, 5% CO₂ for 30 minutes in the presence of the already described growth medium.

5) The supernatant was discarded and further digestion affected by collagenase II (0.8 mg/mL) for 4h at 37°C, 5% CO₂ again with the same growth medium

6) The digested tissue was allowed to settle and the supernatant containing the cells was collected and centrifuged (1500g) for 10m at 4°C

- Cells were seeded in 4 well culture plates at a density of 4000 cell/cm²
- Culture medium consisted of an equal mix of Ham’s F12: DMEM supplemented with a range of growth factors

<table>
<thead>
<tr>
<th>Group</th>
<th>Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F12:DMEM, 2% FBS</td>
</tr>
<tr>
<td>2</td>
<td>F12:DMEM, 10% FBS</td>
</tr>
<tr>
<td>3</td>
<td>F12:DMEM, FGF2 (1ng/mL), TGF-β(1ng/mL), 2% FBS</td>
</tr>
<tr>
<td>4</td>
<td>F12:DMEM, FGF2 (1ng/mL), TGF-β(1ng/mL), ITS(10µg/mL)</td>
</tr>
<tr>
<td>5</td>
<td>F12:DMEM, FGF2(1ng/mL), TGF-β(1ng/mL), ITS(10µg/mL) +2% FBS</td>
</tr>
<tr>
<td>6</td>
<td>F12:DMEM, FGF2(1ng/mL), TGF-β(1ng/mL), ITS(10µg/mL) +10% FBS</td>
</tr>
<tr>
<td>7</td>
<td>F12:DMEM, ITS, 2% FBS</td>
</tr>
<tr>
<td>8</td>
<td>F12:DMEM, ITS, 10% FBS</td>
</tr>
</tbody>
</table>

- Each medium was also supplemented with ascorbic acid(50µl/ml), penicillin (100 µl/ml), streptomycin (100µl/ml) and L-glutamine (200mM)
- Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere for periods of up to 40 days. The medium was changed 2-3 time per week
- Cells were examined for morphological changes daily by phase microscopy
- When cells reached 70%-90% confluency, they were enzymatically detached with trypsin for ~2 min (0.25% w/v) in Ca²⁺ and Mg²⁺ free PBS
<table>
<thead>
<tr>
<th>Markway, B. D., Cho, H. &amp; Johnstone, B.</th>
<th>Healthy chondrocytes from cadaver femoral condyles</th>
<th>Monolayer expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy chondrocytes from cadaver femoral condyles</td>
<td>OA chondrocytes taken from patients undergoing total joint replacement surgery</td>
<td>Chondrocytes were seeded at a density of 7,000 cells per cm² and expanded in monolayer culture in a standard tissue culture incubator under normoxia / 5% CO₂</td>
</tr>
</tbody>
</table>

### Pellet Culture

- Chondrocytes were redifferentiated between 1<sup>st</sup> and 3<sup>rd</sup> passage in a serum-free “chondrogenic induction medium.”
- Medium consisted of high glucose DMEM, TGF-β1 (10 ng/ml), dexamethasone (10⁻⁷), ascorbic acid 2-phosphate (37.5µg/ml), sodium pyruvate (1mM), L-proline (40 µg/mL), 1 x ITS and antibiotics (as before)
- Pellets were formed by centrifuging 1x10⁵ cells at 500g in medium (250µl) and maintained in a hypoxic chamber set a 2% oxygen
- Medium was changed every 2-3 days.

<table>
<thead>
<tr>
<th>1) Samples were taken from the condyles and finely minced</th>
<th>Harvested chondrocytes were washed and re-suspended in PBS before total cell count and viability assessment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2) Digestion was carried out with protease (1% w/v) from Streptomyces Griseus in the presence of low glucose DMEM and penicillin-streptomycin(1%) for 1h at 37°C</td>
<td>Chondrocytes from each medium were passaged 3 times and re seeded at the same starting density and culture media</td>
</tr>
<tr>
<td>3) After 1h protease was substituted for collagenase II (1,300 U/mL) in DMEM antibiotics for 3h/ 37°C</td>
<td></td>
</tr>
<tr>
<td>4) The suspension was passed through a strainer (40-µm) and centrifuged at 500 x g for 5m</td>
<td></td>
</tr>
<tr>
<td>5) Cells were then resuspended in low glucose DMEM w/ FBS (10%) and antibiotics ready for plating</td>
<td></td>
</tr>
</tbody>
</table>

| 7) The pellet was washed three times with PBS or serum-free medium and resuspended. Cell suspensions were passed through a nitex nylon filter (70mm) into serum-containing medium. | |
7.3 Conclusion from Survey

The search indicated that the media composition remains relatively unexplored although not without exception. The use of a high nutrient medium such as DMEM or Coon’s Modified Ham’s F12 in conjunction with fetal calf serum (or autologous serum in ACI protocol) and ascorbic acid derivatives is broadly encompassing of the typical approach. The use of serum appears to be the standard and represents one possible factor in phenotype modification, especially in ACI cases where serum composition may be affected by lifestyle choices or underlying disease. To address this investigators from the University of Genova (P Giannoni et al., 2005) suggested a defined medium protocol. Removal of serum from the expansion regime and replacement with a total defined medium that uses carefully controlled amounts of appropriate growth factors and cytokines to maintain chondrogenic capacity offers an advantage, especially from a manufacturing perspective that maybe look to employ allogenic cell lines. The use of serum in current regimes is preferential because it is cost-effective, whereas the addition of exogenous growth factors and cytokines are comparatively expensive. For the work to be of any practical value identification of cost management opportunities will be paramount.

Extensive work by Paolo Giannoni et al. (2005) proposed a defined medium and the study brought about several other points of note. Firstly, serum-free conditions promoted rapid dedifferentiation of the chondrogenic phenotype in human chondrocytes compared to serum conditions yet reportedly provided superior results (defined by COL2 and aggrecan expression) when redifferentiated in 3D culture. The defined medium also boosted the proliferative capacity of the chondrocytes, reaching a 16 fold increase in population in 35 days. The authors stated that, in contrast, the same increase in cell number takes three to four months in serum. The study also raised questions about the role of TGF-β1 in sustaining
chondrogenic potential during expansion which with the same concern reiterated in a further study (R Narcisi et al., 2012). The work by Giannoni presents itself as an appropriate foundation on which to base the initial screening experiment around with other literature sources providing supplementary angles. Interestingly the media optimisation study by Enochson et al. (2012) found addition of an appropriate amount of TGF-β1 was beneficial. The TGF β series appears to have a duality in chondrocyte culture that is not yet fully understood.

The reoccurring theme across the literature is that the expansion of the cells to sufficient numbers is not the limiting factor but rather retaining the original phenotype of the cells during the expansion. To elaborate, Biterberg et al. (1994) would seed defects with an average size of 3.1 cm² with 2.6 – 5 million cells. Following on from this Genzyme recommend seeding their expanded chondrocytes at approximately 2 million cells per cm² which is fairly representative of recommendations from other commercial outfits (C B Foldager et al., 2012). This level of expansion is easily achievable using standard T-flask culture. So the challenge can be defined as finding a way to preserve the quality of the cells while finding ways to reduce the cost of the manufacturing.

8.0 Study Objectives

The first objective is to isolate and cryopreserve a suitable cell strain for all subsequent experiments. Animal or human articular chondrocytes taken from disease-free joints are all acceptable sources and although the preference is for human chondrocytes circumstance will dictate which is used. Cell strain isolation will be based on existing literature accounts. The effect of expanding the chondrocytes using a conventional serum-
containing medium and an in-house defined medium adapted from the literature will be assessed along with the effect of feeding frequency. Culture periods will be kept short as to avoid introducing extra variables that may be incurred during passaging. The specific metabolic fluxes of glucose, glutamine, lactate and ammonia will be measured in order to understand a) the nature of rate limiting event and b) to investigate if any interesting behaviours can be observed that may warrant further investigation as a potential marker of quality. Alongside this, some conventional methods of assessing quality in chondrocytes will be carried out. Specifically, SOX-9, 6 and 5 transcripts will be determined using RT-qPCR. Once rate-limiting events have been identified a screening experiment will be carried out to identify how the selected culture parameters directly affect the growth rate and SOX transcript expression and how they interact with other parameters. The nature of the work after this point will depend on the results but the ultimate aim is to time resolve, where applicable, the addition of the culture components to the medium as to create a finely controlled expansion environment that balances the need to achieve maximum cell volumes with cell quality in a cost-efficient manner.
CHAPTER B: METHODOLOGY

This chapter gives an account of the materials and methods used in the experiments described in this thesis.

1.0 Cell Culture Medium, Wash Solutions, and Fibronectin Compositions.

A variety of culture mediums and wash mixtures were used in the various experiments described. The tables below outline the components of the mediums and their composition.

1.1 PBS with 3% Antibiotic & Antimycotic (AA)* (100mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS - Ca²⁺ / - Mg²⁺</td>
<td>Lonza</td>
<td>17-516F</td>
<td>RT</td>
<td>N/A</td>
<td>97mL</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic (100x)</td>
<td>Thermo Fischer Scientific</td>
<td>15240062</td>
<td>-20°C</td>
<td>3% v/v</td>
<td>3mL</td>
</tr>
</tbody>
</table>

* Shelf life of solution is seven days at 2-8°C
** Penicillin (10,000 units/mL), Streptomycin (10, 000 µg/mL and Amphotericin B (25 µg/mL)
### 1.2 PBS with 1% Antibiotic & Antimycotic (AA)* (100mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS - Ca²⁺ / - Mg²⁺</td>
<td>Lonza</td>
<td>17-516F</td>
<td>RT</td>
<td>N/A</td>
<td>99mL</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic (100x) **</td>
<td>Thermo Fischer Scientific</td>
<td>15240062</td>
<td>-20°C</td>
<td>1% v/v</td>
<td>1mL</td>
</tr>
</tbody>
</table>

* Shelf life of solution is seven days at 2-8°C  
** Penicillin (10,000 units/mL), Streptomycin (10, 000 µg/mL and Amphotericin B (25 µg/mL)

### 1.3 Pronase E Digestion Medium*** (50mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM:F12</td>
<td>Thermo Fischer Scientific</td>
<td>17-516F</td>
<td>RT</td>
<td>N/A</td>
<td>84mL</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic (100x) **</td>
<td>Thermo Fischer Scientific</td>
<td>15240062</td>
<td>-20°C</td>
<td>1% v/v</td>
<td>1mL</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>Thermo Fischer Scientific</td>
<td>10270098</td>
<td>-20°C</td>
<td>10% v/v</td>
<td>10mL</td>
</tr>
<tr>
<td>GlutaMAX™ (200mM)</td>
<td>Thermo Fischer Scientific</td>
<td>35050061</td>
<td>RT</td>
<td>5mM</td>
<td>2.5mL</td>
</tr>
<tr>
<td>HEPES (1M)</td>
<td>Thermo Fischer Scientific</td>
<td>15630056</td>
<td>2-8 °C</td>
<td>25mM</td>
<td>2.5mL</td>
</tr>
<tr>
<td>Pronase E (3.5 U mg⁻¹)</td>
<td>Sigma Aldrich</td>
<td>P5147-1G</td>
<td>-20°C</td>
<td>70 U ml⁻¹</td>
<td>1g</td>
</tr>
</tbody>
</table>

* Digestion medium divided into 5mL aliquots and frozen (-20°C)
† Medium passed through sterile filter before use (Nalgene™ Rapid-Flow™ Sterile, Thermo Fischer
Scientific, catalogue number: 169-0045)
** Penicillin (10,000 units/mL), Streptomycin (10,000 µg/mL) and Amphotericin B (25 µg/mL)

1.4 Collagenase II Digestion*† Medium (200mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM:F12</td>
<td>Thermo Fischer Scientific</td>
<td>17-516F</td>
<td>RT</td>
<td>N/A</td>
<td>68mL</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic (100x)</td>
<td>Thermo Fischer Scientific</td>
<td>15240062</td>
<td>-20°C</td>
<td>1% v/v</td>
<td>2mL</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>Thermo Fischer Scientific</td>
<td>10270098</td>
<td>-20°C</td>
<td>10% v/v</td>
<td>20mL</td>
</tr>
<tr>
<td>GlutaMAX™ (200mM)</td>
<td>Thermo Fischer Scientific</td>
<td>35050061</td>
<td>RT</td>
<td>5mM</td>
<td>5mL</td>
</tr>
<tr>
<td>HEPES (1M)</td>
<td>Thermo Fischer Scientific</td>
<td>15630056</td>
<td>2-8°C</td>
<td>25mM</td>
<td>5mL</td>
</tr>
<tr>
<td>Collagenase II</td>
<td>Thermo Fischer Scientific</td>
<td>17101015</td>
<td>2-8°C</td>
<td>300U ml⁻¹</td>
<td>218.18mg</td>
</tr>
</tbody>
</table>

* Digestion medium divided into 40mL aliquots and frozen (-20°C)
† Medium passed through sterile filter before use (Nalgene™ Rapid-Flow™ Sterile, Thermo Fischer
Scientific, catalogue number: 169-0045)
** Penicillin (10,000 units/mL), Streptomycin (10,000 µg/mL) and Amphotericin B (25 µg/mL)
## 1.5 FBS Expansion Medium with Antibiotics & Antimycotic*† (500mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM:F12</td>
<td>Thermo Fischer Scientific</td>
<td>17-516F</td>
<td>RT</td>
<td>N/A</td>
<td>373mL</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic (100x) **</td>
<td>Thermo Fischer Scientific</td>
<td>15240062</td>
<td>-20°C</td>
<td>1% v/v</td>
<td>2mL</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>Thermo Fischer Scientific</td>
<td>10270098</td>
<td>-20°C</td>
<td>10% v/v</td>
<td>50mL</td>
</tr>
<tr>
<td>GlutaMAX™ (200mM)</td>
<td>Thermo Fischer Scientific</td>
<td>35050061</td>
<td>RT</td>
<td>5mM</td>
<td>12.5mL</td>
</tr>
<tr>
<td>HEPES (1M)</td>
<td>Thermo Fischer Scientific</td>
<td>15630056</td>
<td>2-8°C</td>
<td>25mM</td>
<td>12.5mL</td>
</tr>
<tr>
<td>2-Phospho-L-ascorbic acid trisodium salt</td>
<td>Sigma Aldrich</td>
<td>49752-10G</td>
<td>RT</td>
<td>0.1mM</td>
<td>16mg in 50mL PBS</td>
</tr>
</tbody>
</table>

* Medium Stored at 2-8 Deg and discarded after seven days. pH approximately 7.25 at 21°C (Degassed). Osmolarity not determined.

† Medium passed through sterile filter before use (Nalgene™ Rapid-Flow™ Sterile, Thermo Fischer Scientific, catalogue number: 169-0045)

** Penicillin (10,000 units/mL), Streptomycin (10, 000 µg/mL and Amphotericin B (25 µg/mL)
### 1.6 FBS Expansion Medium*† (500mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM:F12</td>
<td>Thermo Fischer Scientific</td>
<td>17-516F</td>
<td>RT</td>
<td>N/A</td>
<td>375mL</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>Thermo Fischer Scientific</td>
<td>10270098</td>
<td>-20°C</td>
<td>10% v/v</td>
<td>50mL</td>
</tr>
<tr>
<td>GlutaMAX™ (200mM)</td>
<td>Thermo Fischer Scientific</td>
<td>35050061</td>
<td>RT</td>
<td>5mM</td>
<td>12.5mL</td>
</tr>
<tr>
<td>HEPES (1M)</td>
<td>Thermo Fischer Scientific</td>
<td>15630056</td>
<td>2-8°C</td>
<td>25mM</td>
<td>12.5mL</td>
</tr>
<tr>
<td>2-Phospho-L-ascorbic acid trisodium salt</td>
<td>Sigma Aldrich</td>
<td>49752-10G</td>
<td>RT</td>
<td>0.1mM</td>
<td>16mg in 50mL PBS</td>
</tr>
</tbody>
</table>

* Medium Stored at 2-8 Deg and discarded after seven days. pH approximately 7.45 at 21°C (Degassed). Osmolarity not determined.

† Medium passed through sterile filter before use (Nalgene™ Rapid-Flow™ Sterile, Thermo Fischer Scientific, catalogue number: 169-0045)

### 1.7 Fibronectin Solution for Colony Isolation*† (10µg/mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS - Ca²⁺ / - Mg²⁺</td>
<td>Lonza</td>
<td>17-516F</td>
<td>RT</td>
<td>N/A</td>
<td>100mL</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>Sigma Aldrich</td>
<td>C5670-100G</td>
<td>RT</td>
<td>1mM</td>
<td>11mg</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>Sigma Aldrich M4880-100G</td>
<td>RT</td>
<td>1mM</td>
<td>20mg</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------</td>
<td>----</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
</tbody>
</table>

| Plasma Derived Bovine Fibronectin (1mg) | Thermo Fischer Scientific 33010018 | 2-8°C | 10µg/mL | N/A |

* Solution divided into 7mL aliquots and stored at -20°C.

† PBS supplemented with calcium and magnesium chloride passed through a sterile filter (Nalgene™ Rapid-Flow™ Sterile, Thermo Fischer Scientific, catalogue number: 169-0045) before using 1mL of the sterile mixture to reconstitute the lyophilized Fibronectin. The reconstituted Fibronectin was stored at 1µg/mL (-20°C) until needed. Dilutions were made by adding the required amount of stock solution to the appropriate volume of calcium and magnesium chloride supplemented PBS.
### 1.8 Defined Expansion Medium*† (100mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Thermo Fischer Scientific</td>
<td>A1443001</td>
<td>RT</td>
<td>N/A</td>
<td>94.95mL</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>Sigma Aldrich</td>
<td>G7021-1KG</td>
<td>RT</td>
<td>1g/L</td>
<td>100mg</td>
</tr>
<tr>
<td>Bovine Serum Albumin (Fraction V)</td>
<td>Sigma Aldrich</td>
<td>A9418-10G</td>
<td>2-8 °C</td>
<td>1% w/v</td>
<td>1g</td>
</tr>
<tr>
<td>Chemically Defined Lipid Mixture 1</td>
<td>Sigma Aldrich</td>
<td>L0288-100ML</td>
<td>2-8 °C</td>
<td>0.5% v/v</td>
<td>0.5mL</td>
</tr>
<tr>
<td>Kao and Michayluk Vitamin Solution (100x)</td>
<td>Sigma Aldrich</td>
<td>K3129-100ML</td>
<td>-20°C</td>
<td>1% v/v</td>
<td>1mL</td>
</tr>
<tr>
<td>L-Glutamine (200mM)</td>
<td>Thermo Fischer Scientific</td>
<td>25030081</td>
<td>2-8 °C</td>
<td>5mM</td>
<td>2.5mL</td>
</tr>
<tr>
<td>ITS (100x)</td>
<td>Sigma Aldrich</td>
<td>I2521-5ML</td>
<td>2-8 °C</td>
<td>1% v/v</td>
<td>1mL</td>
</tr>
<tr>
<td>Dexamethasone‡</td>
<td>Sigma Aldrich</td>
<td>D4902-100MG</td>
<td>-20°C</td>
<td>10^{-8} M mol/L</td>
<td>40µL</td>
</tr>
<tr>
<td>Human Recombinant PDGF-BB‡</td>
<td>R&amp;D Systems</td>
<td>220-BB-010</td>
<td>-80°C</td>
<td>10ng/mL</td>
<td>50 μL</td>
</tr>
<tr>
<td>Human Recombinant FGF2‡</td>
<td>R&amp;D Systems</td>
<td>233-FB-025/CF</td>
<td>-80°C</td>
<td>10ng/mL</td>
<td>8 μL</td>
</tr>
<tr>
<td>Human Recombinant EGF‡</td>
<td>R&amp;D Systems</td>
<td>236-EG-200</td>
<td>-80°C</td>
<td>10ng/mL</td>
<td>50 μL</td>
</tr>
<tr>
<td>HCl (1N)</td>
<td>Sigma Aldrich</td>
<td>H9892-100ML</td>
<td>RT</td>
<td>Variable</td>
<td>Variable</td>
</tr>
</tbody>
</table>
Medium Stored at 2-8 Deg and discarded after seven days. pH varies depending on the extent of degassing induced by vacuum filtration. Target pH approximately 7.50. Higher pHs can be reduced by adding HCl - 100μladdition to 100mL total volume achieves an approximate drop of 0.2. Osmolarity =

Medium passed through sterile filter before use (Nalgene™ Rapid-Flow™ Sterile, Thermo Fischer Scientific, catalogue number: 169-0045)

See 1.1.2.2 for reconstitution explanation

**1.9 Defined Resuspension Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in mixture</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Thermo Fischer Scientific</td>
<td>A1443001</td>
<td>RT</td>
<td>N/A</td>
<td>100mL</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>Sigma Aldrich</td>
<td>G7021-1KG</td>
<td>RT</td>
<td>1g/L</td>
<td>100mg</td>
</tr>
<tr>
<td>Bovine Serum Albumin (Fraction V)</td>
<td>Sigma Aldrich</td>
<td>A9418-10G</td>
<td>2-8°C</td>
<td>1% w/v</td>
<td>1g</td>
</tr>
<tr>
<td>HCl (1N)</td>
<td>Sigma Aldrich</td>
<td>H9892-100ML</td>
<td>RT</td>
<td>Variable</td>
<td>Variable</td>
</tr>
</tbody>
</table>

* Medium Stored at 2-8 Deg and discarded after seven days. pH varies depending on the extent of degassing induced by vacuum filtration. Target pH approximately 7.50. Higher pHs can be reduced by adding HCl - 100μladdition to 100mL total volume achieves an approximate drop of 0.2. Osmolarity =

† Medium passed through sterile filter before use (Nalgene™ Rapid-Flow™ Sterile, Thermo Fischer Scientific, catalogue number: 169-0045)
### 1.10 Growth Factor and Dexamethasone Reconstitution Description

<table>
<thead>
<tr>
<th>Component</th>
<th>Carrier Volume</th>
<th>Albumin</th>
<th>HCl (IN)</th>
<th>Aliquot Volume</th>
<th>Aliquot Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant Human TGF-β1 (2µg)</td>
<td>PBS - 100 µL</td>
<td>10mg (0.1%w/v)</td>
<td>4mM</td>
<td>50µL</td>
<td>20 µg/mL (1 µg)</td>
</tr>
<tr>
<td>Recombinant Human TGF-β3 (2µg)</td>
<td>PBS - 100 µL</td>
<td>10mg (0.1%w/v)</td>
<td>4mM</td>
<td>50µL</td>
<td>20 µg/mL (1 µg)</td>
</tr>
<tr>
<td>Human Recombinant PDGF-BB (10µg)</td>
<td>PBS - 100 µL</td>
<td>10mg (0.1%w/v)</td>
<td>4mM</td>
<td>50µL</td>
<td>100 µg/mL (1 µg)</td>
</tr>
<tr>
<td>Human Recombinant FGF2 (25µg)</td>
<td>PBS - 500 µL</td>
<td>50mg (0.1%w/v)</td>
<td>NA</td>
<td>8 µL</td>
<td>125 µg/mL (1 µg)</td>
</tr>
<tr>
<td>Human Recombinant EGF (200µg)</td>
<td>PBS - 1000 µL</td>
<td>50mg (0.1%w/v)</td>
<td>NA</td>
<td>5 µL</td>
<td>200 µg/mL (1 µg)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Absolute Ethanol -</td>
<td>N/A</td>
<td>4mM</td>
<td>40 µL</td>
<td></td>
</tr>
</tbody>
</table>
2.0 Method for Isolating α5-Integrin Receptor-Expressing Cell Sub-Population from Bovine Metacarpophalangeal Joints

2.1 Tissue Explant

Fresh metacarpophalangeal joints were obtained from twenty to twenty-four-month-old cows at the FOYLE Melton Mowbray animal processing site under an AB117 home office permission license. Four joints were used in developing the explant and banking method. The clonal chondrocytes used in the described experimental chapters were derived from a single joint unless stated otherwise. The animal tag and kill number was recorded in the event the provenance of the material need to be evaluated later. Harvested tissue was packed on ice and transported back to the University for processing. The explant procedure commenced within two hours of the animal sacrifice. The tissue joint was gently washed with detergent to remove soil and grease before drying followed by removal of the hair with clippers. The tissue was placed in a dissection tray and, using a scalpel and the appropriate PPE; incisions are made to reveal the ligaments that support the joint capsule. Severing the ligaments exposed the joint capsule. The joint was transferred to a sterile dissection tray in a laminar flow hood. PBS 3% antibiotic-antimycotic (1.1), pre-warmed in a water bath, (37°C, minimum 20 minutes) was pipetted over the cartilage surface to maintain a moisture layer. The cartilage was scraped off the surface using a sterile scalpel and the shavings placed into a Petri dish containing PBS containing 3% AA (1.1).
2.2 Primary Cell Isolation.

The following procedure was conducted under sterile conditions. After the harvest was completed, an amount of wet tissue was weighed out and placed into a centrifuge tube (50mL) while maintaining sterility. The Pronase E digestion medium (1.3) (pre-warmed to 37°C) was added to the tissue at a ratio of 1mL per 100mg of tissue. The mixture was placed in a water bath (37°C) and gently agitated every ten minutes. The digestion was halted after one hour by removing the digestion medium with centrifugation (300g/5min, room temperature) and removal of the supernatant with an aspirator. The mixture was washed with PBS containing 3% AA (1.1). The tissue was centrifuged again (300g/5min) and the PBS wash removed and replaced with the Collagenase II Digestive Medium (1.4, pre-warmed 37°C) at a ratio of 1mL per 100mg of tissue. The mixture was placed in a water bath (37°C) and gently agitated every twenty minutes. After three hours the tube was removed from the water bath and centrifuged (300g/5min) before washing with 10mL PBS containing 1% AA (1.2, pre-warmed to 37°C). The PBS was aspirated off and the wash step repeated. After resuspending, the digestion mix was collected with a stripette (10mL) and passed through a filter (100µm) to remove undigested matrix and debris. The filtrate was collected and centrifuged (300g/5min) before removing the supernatant. The cell pellet was re-suspended in 5-10mL of growth medium before cell counting. Cell counts were performed using a hemocytometer following the description in 7.1.
2.3 α5-Integrin Receptor-Expressing Cell Strain Separation

The cell subpopulation was isolated using the differential adhesion to Fibronectin assay described by Dowthwait et al. (2004). After performing the cell counts described in 7.1, six-well (Thermo Fischer Nunc™ Treated, Cat. No. 140675) plates that were pre-coated overnight with Bovine Fibronectin (1.7, diluted with PBS to give a final concentration of 1µg/cm²). The next morning the Fibronectin solution was aspirated from the wells prior to cell seeding. Densities of 104 cells/cm², 52 cells/cm² and 26 cells/cm² were seeded and the volume of medium made up to a total 1.5mL (1.1.1.5). One well was not subjected to Fibronectin pre-coating to confirm fibronectin-dependent attachment. Cells were allowed to adhere to the surface for twenty minutes in a humidified incubator (5% CO₂/ 37°C) before aspirating off the medium and unattached cells. Each well was replenished with 2mL of growth medium (1.5). Colonies, defined as clusters of cells sixty-four or more, were grown over a period of seven days. Medium was replenished after approximately 80 hours.

2.4 Colony Isolation

Colonies were typically judged to be ready for isolation once reaching a size of ~ two hundred cells or approximately eight population doublings. Colonies to be harvested were located using an inverted microscope and a mark placed on the outer surface of the well plate to denote the target area for cloning disks. Under sterile conditions, cloning disks (Sigma-Aldrich, Cat. No., Z374431-100EA) were soaked in TrypLE Express (Thermo Fischer, Cat. No., 12604021) and manoeuvred onto the premarked colony using suitable forceps. Care is required not to streak the disk across the plate or over saturate the disk with the enzyme as to avoid the possibility of bleeding colonies together. The disk was left in place for five minutes before transferring to a fresh well plate containing 2mL of growth medium (1.6, pre-warmed
to 37°C). The disk was gently agitated once in the medium to encourage migration of cells to the tissue culture plastic.

### 2.5 Mycoplasma Testing and Monitoring for Adventitious Agents

After the Colony Isolation step, cells were expanded over five days before detachment and reseeding into T25 flasks (Thermo Fischer Nunc™ Treated, Cat. No. 136196) at a density of 3000 cells/cm². Cells surplus to requirement were subjected to mycoplasma testing using a MycoAlert™ test kit (Lonza, Cat. No., LT07-118) according to manufacturer instructions. Samples that returned a negative group were advanced the cryopreservation stage.

### 2.6 Cryopreservation and Formation of Working-Cell Bank

The initial cell expansion yields between two to four million cells per colony isolation (approximately twenty-one to twenty-two population doublings). After this expansion period, cryopreservation was implemented. A master cryomix was made by adding one part DMSO (Sigma-Aldrich, Cat. No., D4540-100ML) to nine parts Fetal Bovine Serum (Thermo Fischer, Cat. No., 10270098). The cryomix was filtered through a compatible 0.2µm filter to achieve sterility. Detached cells were equilibrated with ambient temperature and placed at a concentration of 1x10⁶ into pre-labeled cryovials containing 1mL of the cryomix. No more than ten minutes were allowed to elapse before placing completed vials in a Cool Cell™ at -80°C. Processed cryovials were kept at -80°C for twenty-four hours before long-term storage in the vapor phase of liquid nitrogen. The expansion of all individual colonies yielded approximately one hundred and twenty vials there formed the basis of the working bank.
3.0 Thawing and Seeding of Cryopreserved Cells for Serum Based Expansions.

A T175 was prepared by adding 28mL of medium (1.6) and allowing to thermally equilibrate in an incubator (37°C). Additional medium was also warmed (37°C). The vial was removed from cryostorage and immediately warmed in a water bath (37°C) until the vial thawed (approximately 90 seconds). Under sterile conditions, 1.5mL of the pre-warmed medium was added to the cryovial and mixed with a gentle pipetting action. The contents of the vial were transferred to a centrifuge tube containing 3.5mL of expansion medium (1.6). DMSO was removed by centrifuging at 250g for 5 minutes and aspirating off the supernatant. The cell pellet was re-suspended in 2mL of expansion medium. A cell count can be performed (7.2) to verify cell number and viability (membrane integrity). The cell suspension was carefully added to the flask containing the pre-warmed medium avoiding bubble formation.

4.0 Thawing and Seeding of Cryopreserved Cells for Defined Based Expansions.

The procedure was as described in 3.0 with the following exceptions. The T175 vessel was pre-coated with 1μg/cm² Fibronectin solution (14mL, 1.7). Once added the T-flask was placed in an incubator (37°C) and a two hour incubation period follows. After 2 hours the excess coating solution was removed and defined medium (1.8) added to the flask and allowed to equilibrate in the incubator for an hour prior to seeding.
5.0 Cell Detachment Procedure for Serum Medium Expanded Cells.

Under sterile conditions, the medium from the vessel was aspirated off and the cells washed with the appropriate volume (see vessel-volume ratio in the table) of PBS (37°C) as to remove traces of serum. TrypLE (37°C) was added to the vessel (see Table 1 for volume), the vessel placed back into an incubator (37°C) and the timer started. When processing multiple vessels, attention was required to limit the variation in exposure time. This was achieved, when working with T-Flasks, by adding the volume of dissociation reagent to the flask in an upright orientation and not allowing the enzyme to contact the cell surface till all flasks were prepared. In well plates were this was not possible the number of wells processed at any one time was limited to twelve so that difference in exposure time to the TrypLE between the wells was negligible. After the appropriate time had elapsed serum containing expansion medium (1.6) was added at a minimum ratio of 1:1.

Table 1 Reagent volumes for various expansion vessels.

<table>
<thead>
<tr>
<th>Expansion Vessel</th>
<th>PBS Wash Volume</th>
<th>TrypLE Volume</th>
<th>Cleavage Time</th>
<th>Quench Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Well Plates</td>
<td>1mL</td>
<td>1mL</td>
<td>7 min</td>
<td>1mL</td>
</tr>
<tr>
<td>T25</td>
<td>5mL</td>
<td>5mL</td>
<td>7 min</td>
<td>5mL</td>
</tr>
<tr>
<td>T75</td>
<td>7mL</td>
<td>7mL</td>
<td>7 min</td>
<td>7mL</td>
</tr>
<tr>
<td>T175</td>
<td>14mL</td>
<td>14mL</td>
<td>7 min</td>
<td>14mL</td>
</tr>
</tbody>
</table>
6.0 Cell Detachment Procedure for Defined Medium Expanded Cells.

The procedure was the same as in 5.0 with the exception of the quenching agent. Instead of using serum-containing medium a defined trypsin inhibitor (Thermo Fischer Scientific, Catalogue Number R007100) was added at a 1:1 ratio.

7.0 Cell Counting Methods

Cell counts were obtained using multiple methods depending on equipment availability. The NucleoCounter® NC-3000™ (Chemotec) is the preferred count platform and accepts three formats, designated: Via-1 Cassette, A2 slides & A8 slides.

7.1 Haemocytometer

The cell suspension was gently vortexed for 10 seconds to ensure mixing. A 50µL sample was taken and mixed with 50µL Trypan Blue solution (Sigma-Aldrich, Cat. No. T8154-100ML) to ascertain cell viability (membrane integrity). The mixture was gently vortexed for 10 seconds before spotting a hemocytometer with a ~20 µL sample. Four squares of the hemocytometer squares are counted obeying count rules using an inverted light microscope. Live cell number and cell viability is calculated using the formula:

\[
\text{Total Live Cells (mL)} = \frac{\text{Live Cells Counted}}{\text{No. of Squares}} \times \frac{\text{Dilution Factor}}{10^4} \\
\]

\[
\text{Cell Viability(\%)} = \left( \frac{\text{Live Cells}}{\text{Total Cells}} \right) \times 100
\]
7.2 Via 1-Cassette

The cell suspension was gently vortexed for 10 seconds to ensure mixing. A 100µL sample is transferred to an Eppendorf™ tube (1.5mL). The cassette tip was offered to the sample and the cell suspension drawn up into the cassette by applying pressure to the button. Cell counts are automatically calculated based on Acridine Orange total staining and DAPI for nonviable staining which are pre-loaded onto the cassettes.

7.3 A2 and A8 Slides

The cell suspension was gently vortexed for 10 seconds to ensure mixing. A 95µL sample was removed and mixed with five µL of solution 13 (pre-mix of DAPI and acridine orange, Chemotec, Catalogue Number 910-3013). The suspension was mixed by pipetting action before loading into the slide chambers (~10 µL per chamber). A2 and A8 slide formats use the same procedure with the difference being the number of individual chambers available.

7.4 Growth Calculations

Population doublings (equation 1) and specific growth rates (equation 2) were calculated using the expressions below

\[
Population Doublings = \frac{1}{\log(2)} \cdot \log \left( \frac{C_x(t)}{C_x(0)} \right)
\]

Equation 1. Population doubling expression where \(C_x(t)\) is the cell number at the end of the growth phase and \(C_x(0)\) is the cell number at time 0.
Specific Growth Rate = \frac{\ln \left( \frac{c_x(t)}{c_x(0)} \right)}{\Delta t}

**Equation 2.** Population doubling expression where \( C_x(t) \) is the cell number at the end of the growth phase, \( C_x(0) \) is the cell number at time 0 and \( \Delta t \) is the difference in time units between \( C_x(t) \) and \( C_x(0) \).

**8.0 Light Microscopy**

Routine inspection of cells, cell imaging and cell counts using a hemocytometer were performed using brightfield microscopy on a Nikon Eclipse Ti inverted microscope (10x and 40x magnification).

**9.0 pH Measurements**

pH readings were obtained using an Accumet™ AB150 pH meter (Fischer Scientific). Before taking readings, a one-point calibration was performed using a pH seven standard (Fischer Scientific, Cat. No. 10457440).

**10.0 Osmolarity Measurements**

Osmolarity was determined using an Osmomat 010 (Gonotec) osmolarity meter. The meter was calibrated before use by normalising with de-ionised water to 0 then calibrated using NaCl calibration standards. The osmeter probe was cleaned with distilled water between uses.
11.0 Metabolite Analysis

Medium to be analysed was removed from the expansion vessel. After mixing by pipetting action, a 1mL sample was taken and placed in an Eppendorf™ tube (1.5mL). The sample was stored at -20°C until processing. The storage conditions were assessed in previous experiments (data not shown) which concluded there was no significant difference in the observed metabolite concentrations from unfrozen samples tested immediately and the same sample tested after one month of storage at -20°C. Frozen vials were thawed at room temperature then vortexed before loading onto a Cedex Bio HD analyser (Roche). The analyser determines analyte concentration using enzymatic–spectrophotometric methods. Table 2 indicates the tests employed by the analyser. Before running, onboard calibration for each test is run and an independent sample containing known amounts of glucose, glutamine and lactate are run to assess the level of agreement between onboard calibration and sample concentration.
Table 2. Consumables for the Cedex Biostation HD and the principle method for metabolite determination.

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>Catalogue Number</th>
<th>Determination Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Bio HT</td>
<td>Roche</td>
<td>06608418001</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>Glutamine Bio HT</td>
<td>Roche</td>
<td>06605800001</td>
<td>Glutamine Hydrolysis to Glutamate</td>
</tr>
<tr>
<td>Calcium Bio HT</td>
<td>Roche</td>
<td>06990100001</td>
<td>NM-BAPTA</td>
</tr>
<tr>
<td>Acetate Bio HT</td>
<td>Roche</td>
<td>06608493001</td>
<td>Lactate to Pyruvate Conversion (IFCC method)</td>
</tr>
<tr>
<td>Lactate Bio HT</td>
<td>Roche</td>
<td>06608485001</td>
<td>Enzymatic oxidation by lactate oxidase</td>
</tr>
<tr>
<td>NH3 Bio HT</td>
<td>Roche</td>
<td>06608515001</td>
<td>Enzymatic amination by glutamate dehydrogenase</td>
</tr>
<tr>
<td>Pyruvate Bio HT</td>
<td>Roche</td>
<td>07299818001</td>
<td>Enzymatic reduction by lactate dehydrogenase with NADH</td>
</tr>
<tr>
<td>Acetate Bio HT</td>
<td>Roche</td>
<td>07044399001</td>
<td>Enzymatic reaction with acetyl CoA synthetase and citrate synthase</td>
</tr>
</tbody>
</table>
11.1 Calculation of Specific Metabolic Fluxes.

Cell normalised consumption or production rates were calculated using equation 3.

\[
Specific \ flux = \left( \frac{\mu}{c_x(0)} \right) \left( \frac{c_{\text{met}}(t) - c_{\text{met}}(0)}{e^{\mu t} - 1} \right)
\]

**Equation 3.** Expression for calculating specific flux rates where \( \mu \) is specific growth rate (day\(^{-1}\)), \( c_x(0) \) is the final cell number, \( c_{\text{met}}(t) \) and \( c_{\text{met}}(0) \) are the final and initial metabolite concentrations (mmol), respectively and \( t \) is time (day).

12.0 RT-q PCR

The account here describes how SOX 9, 6 and 5 mRNA transcripts were quantified using Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR). The stages are broken down into Cell Archiving, RNA extraction, RNA quantification and quality control, Reverse Transcription, qPCR and Normalisation and copy number determination. The methods described were developed by qStandards PCR and assays were run at their site in Middlesex.

12.1 Cell Archiving

The cell suspension obtained after detachment was mixed with RNAlater™ RNA Stabilization Solution (Qiagen, Catalogue Number, 76104) at a ratio of 1:5 in an Eppendorf™ tube. The sample was stored at \( -20^\circ \text{C} \) ready for RNA extraction at a later date. Alternatively, this step was excluded, and RNA extraction performed immediately after cell detachment.
12.2 RNA Extraction and Purification

RNA extraction and purification was achieved using RNeasy Mini Kit (Qiagen, Catalogue Number, 74104). The cell suspension (1x10^7 max) was lysed by aggressive vortexing for 5 minutes. The ethanol supplemented lysis buffer was added to the lysate then loaded onto the affinity column. The column was washed three times with distilled water and a centrifuge (30 seconds, 200g) to move the eluent out the column at each wash stage. After the initial wash steps, a DNase wash was added to remove genomic DNA contamination followed by a centrifugation step (30 seconds, 200g). RNA was eluted from the column using the DNase/RNase free water by the addition of 5µl directly to the surface of the column. The product was eluted with a centrifugation step (30 seconds, 200g) into a sterile RNase-free collection tube. Samples were frozen before total RNA quantification and quality check at a later date.

12.3 RNA Quantification

RNA quantification was performed using a NanoDrop 2000 UV-Vis spectrophotometer. The machine was blanked using DNase/RNase water before loading of thawed samples. The 260/280, 260/230 absorbance’s were recorded along with the estimated quantity. Total RNA integrity was determined using an Agilent 6000 Bioanalyzer and Nano Chips (Agilent, 5067-1511). The ladder and samples were loaded as per instructions. The electropherogram was interrogated for 18 and 28s ribosomal peaks. Samples with a RIN higher than 9 were accepted and submitted for reverse transcription.
12.4 Reverse Transcription

The total RNA was reverse transcribed using the Qiagen Quantitect reverse transcription kit (Qiagen, Cat. No., 205313). Where possible 500ng of the total RNA was used in a 10µl reaction as per manufacturer instructions. Typically a reverse transcription duplicate was performed every five samples. Completed reactions were diluted tenfold and supplemented with 0.5µg/mL tRNA in water. cDNA was stored at -20°C until ready to perform the qPCR reaction.

12.5 Primer Design

Custom primers for SOX-9, 6 and 5 were designed using the NCBI Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were designed to be intro spanning as to avoid the amplification of genomic DNA and produced by Sigma-Aldrich using the pure and simple primer service. Primers for the endogenous controls for Beta-Actin (ACTB), Beta-2-Microglobulin (B2M) and Ribosomal Protein L13 (RPL13) were obtained as pre-designed primers (Sigma-Aldrich, Cat. No., KSPQ12012). The primer sequences are outlined in table 3.
Table 3. Forward and reverse primers for genes of interest and endogenous control genes used in the qPCR reactions

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Direction</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX-9</td>
<td>FWD</td>
<td>AACAAGCCGCACCTCAAGCG</td>
</tr>
<tr>
<td></td>
<td>REV</td>
<td>GCCTGGCCGTTCTTCAACGA</td>
</tr>
<tr>
<td>SOX-6</td>
<td>FWD</td>
<td>TCTCCGTCAACACAGACATCCAG</td>
</tr>
<tr>
<td></td>
<td>REV</td>
<td>GGGTAGTTATCAGCTTTGTTATG</td>
</tr>
<tr>
<td>SOX-5</td>
<td>FWD</td>
<td>TTCCTTGACATGAATGTGATGCA</td>
</tr>
<tr>
<td></td>
<td>REV</td>
<td>CTTTGCCTCTTCAGGGTATTC</td>
</tr>
<tr>
<td>ACTB</td>
<td>FWD</td>
<td>CAAGGCAACCGTGAGAA</td>
</tr>
<tr>
<td></td>
<td>REV</td>
<td>GTACATGGCGAGGGGTGTGG</td>
</tr>
<tr>
<td>B2M</td>
<td>FWD</td>
<td>CACCCCGATGAAATGTA</td>
</tr>
<tr>
<td></td>
<td>REV</td>
<td>CAGGTCTGACTGCTCCGATT</td>
</tr>
<tr>
<td>RPL13</td>
<td>FWD</td>
<td>TCCACCACCTATGACAAG</td>
</tr>
<tr>
<td></td>
<td>REV</td>
<td>GAGTAGGCTTCAGACGACA</td>
</tr>
</tbody>
</table>

12.6 Preparation of Standards

Separate standards for each of the three SOX genes were prepared from a PCR product. The target cDNA PCR product was run and separated using pre-cast agarose E-gels (Thermo Fischer, Cat. No., G661818). The electrophoresis and visualisation was carried out on the E-gel Power Snap Electrophoresis device (Thermo Fischer, Cat. No., G8100). The intended product was identified using an E-Gel 1Kb plus DNA ladder (Thermo Fischer, Cat. No., 10488090). Once the cDNA product was identified, a slice of the gel containing the
band was excised and the gel dissolved and purified using the PureLink Quick Gel Extraction Kit (Thermo Fischer K2100-12). Purified products were stored in the elution buffer at -20°C until ready for use in PCR reactions.

12.7 Quantitative Polymerase Chain Reaction

Two microliters of the previously reverse transcribed cDNA were amplified in a 10µl reaction using the Qiagen Quantifast SYBR Green master mix (Qiagen, Cat. No., 204154) with final primer concentrations of 500 nmol/L. A no template control was set up with 0.5µg/mL of tRNA. A seven-point dilution of the previously prepared standards ranging from 10000000 to 10 copies were included in duplicate for each gene with every run. This gave the standard curve for copy number determination. The reactions were pipetted using a robot to reduce pipetting error (Qiagility) and loaded into the Rotor-Gene qPCR platform. Amplification parameters for the SOX genes were 95°C for 5 mins, followed by 40 cycles of 95°C for 10 seconds and 60°C for 20 seconds. Due to primer-dimer formation in the SOX-6 assay cycling conditions were altered to measure the fluorescence at 81°C due to the primer dimers melting at 76°C. The cycles were identical for the endogenous reference genes with the exception the annealing and extension temperature was reduced to 57°C. Melt curves were run to check for product specificity. All melt curves produced a single peak with the exception of SOX-6.

12.8 Copy Number Determination and Data Normalisation.

Copy number were determined from the standard curve and calculated directly by Rotor-Gene software. To account for differences in RNA quantity, reverse transcriptase
efficiency and other uncontrolled variables the gene of interest copy number was normalised to endogenous reference genes. The copy number of the three reference genes, ACTB, B2M, and RPL13, were calculated from their corresponding standard curves and used to calculate the geometric means (average copy number of all three genes). The geometric mean for each sample was divided by 10,000 before calculating the grand geometric mean (the average geometric mean from all samples). The geometric mean divided by 10,000 was then further divided by the grand geometric mean to arrive at the normalisation factor. The determined copy number for each sample is then divided by the calculated normalisation factor to give the normalised copy number.

13.0 Design of Expert and Design of Experiment setup

Nine factors (Table 4) were screened for their impact on growth rate, metabolic function, and SOX gene expression. As discussed earlier, the factors were evaluated in table 1 against a wider panel of candidates. The nine selected candidates were fed into a ½ fraction fractional factorial experiment with three centre points. Due to the erratic nature occasionally encountered and the generally sensitive nature of cell culture it was decided that replication of edge points was a sensible measure. Replication of edge points decreases the leverage of the individual run has over the experiment and also increases the power of the experiment. Factorial type experiments offer the advantage of built-in replication which means the replicates required to generate the desired power to observe a given effect size may be less than those required by non factorial designs. The 9-4 fractional factorial experiment breaks down into 32 individual runs, with the addition of four replications of each edge point and three centre points a total size of 132 runs is reached, which is in the region of the suggested
sample size concluded from a priori power analysis generated using free-to-use GPower software.

13.1 Design of Experiment Software and Setup

The fractional factorial design matrix was generated using Design-Expert V7.0 by Stat-Ease. In order to access the design options first, a “new design” was selected from the file drop down tab. Next, the “Factorial” tab was selected and subsequently the “2-level factorial” option. A nine-factor, 32-run which corresponds to a 9-4 resolution IV design was selected. At the bottom of the page, the “replicate number” was increased from 1 to 4 and the number of centre points increased from 0 to 3. The “continue” button generates the design alias list and cycling through the “continue” options allows the generic design matrix on the final page to be customised with the appropriate name tags for factors and outputs. The matrix generated was used to set the experimental conditions for each run. The returned experimental data was inputted into design matrix. Completing all the data fields allowed the analysis tab for each factor to be explored and the data output interrogated.

13.2 Nuisance Experimental Factors

A concern of the experiment was the potential impact of factors that were not directly under investigation but could have a profound effect on the dependent variable. The three factors of most concern were pH, variation between clones, and variation in consistency of feed mediums between feeds. The rapid degassing of culture medium that is outside of a 5% CO₂ environment was a major problem encountered in the earlier chapter B studies, and with
the addition of HEPES deemed detrimental to the cell line a different strategy had to be adopted. Formulated mediums were allowed to rest for overnight to completely equilibrate with their surroundings. The pH at this time was typically about 8.4. Each total medium was titrated with 1N HCl to lower the pH to around 7.4. The medium was then transferred to the well plates and placed in an 5% CO$_2$ incubator prior to seeding. Before seeding cells into their respective well plates, the pH was rechecked to confirm that the buffering capacity offered by the 5% CO$_2$ atmosphere was sufficient and that the pH was holding around 7.4.

The scale of the experiment has made the inclusion of biological (clonal) replication difficult to achieve. Previous results have shown that clones typically behave in a similar manner as each other although the magnitude of a given output measurement may vary from clone to clone. Quite often the variation arising from technical replication can eclipse variation arising from biological replication. With this in mind, it was decided that the screening experiment would be run with just one clonal replicate. A single vial of clonal chondrocytes was thawed according to protocol.

Extended durations of culture demand replenishment of the growth medium. Variation of growth medium formulations made in-house, and the decomposition of proteins in stored medium could have an unknown and unquantifiable impact on output measurements. To mitigate the risk from this, the experiment was run in fed-batch configuration and the culture period limited to five days as to avoid necessary medium exchanges.
13.3 Experiment Setup

The nine factors were assigned practical concentrations (Table 4). A single vial of clonal chondrocytes was thawed and pre-expanded over six days in the defined medium in order to reach appropriate cell numbers for the experiment.

Growth medium was made up to complement each of the 32 experimental runs and added to a well plate that had the appropriate concentration of fibronectin for that run. After a pH adjustment period and final check (as described in 13.2) cells were added to the well plates to achieve the required seeding density for that run. The seed order for the experiment was randomised as was the order of cell sacrifice after the five day culture period in order to try and minimise time induced systematic bias.

**Table 4.** Candidates for screening paired with their respective coded and actual values

<table>
<thead>
<tr>
<th>Factor</th>
<th>Name</th>
<th>Units</th>
<th>Low Actual</th>
<th>High Actual</th>
<th>Low Coded</th>
<th>High Coded</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>D - Glucose</td>
<td>g/L</td>
<td>0.5</td>
<td>2</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>L - Glutamine</td>
<td>mmol</td>
<td>2.5</td>
<td>10</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>ITS</td>
<td>ml/L</td>
<td>5</td>
<td>20</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>Fibronectin</td>
<td>ug/cm²</td>
<td>0.5</td>
<td>2</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>TGFβ1</td>
<td>%</td>
<td>0</td>
<td>10</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>PDGF-BB,</td>
<td>ng/mL</td>
<td>0</td>
<td>20</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>FGF2.</td>
<td>ng/mL</td>
<td>0</td>
<td>20</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>EGF</td>
<td>ng/mL</td>
<td>0</td>
<td>20</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>J</td>
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<td>cells/cm²</td>
<td>1500</td>
<td>6000</td>
<td>-1</td>
<td>1</td>
</tr>
</tbody>
</table>
13.4 Fractional Factorial Screening Experiment Results

Data from each output field is displayed as a Pareto chart. The chart includes the t-value limit for significance. The chart shows effects in one of two colours. Orange colours, or “positive effects”, show that the highest response for the observed output was achieved when that factor was in the higher of the two experimental levels. Conversely, blue colours, or negative effects”, show that the highest response for the observed output was achieved when that factor was in the lowest of the two experimental levels. To account for inflation in family-wise error rate when making multiple comparisons a correction factor was included. The Bonferroni limit (shown in orange) is the threshold for significance in these experiments. Anything above the threshold can be considered as having a statistically significant impact on the measured output. Two-factor interactions were predominately but not exclusively aliased with three factors interactions; any confounding issues are highlighted in the discussion if a two-factor interaction is of interest. Three-factor interactions, due to the level of aliasing, were disregarded. To assess the confidence in the returned data a number of diagnostic tests are presented to support the Pareto chart. These charts are; the normal probability plots to assess whether residuals follow a normal probability, a line fitted residuals versus run order to establish if any time-related bias during processing significantly influenced the result, externally studentized residuals versus run order to highlight potential outliers and leverage to highlight any runs that are having a disproportionate influence on the fitted model.
CHAPTER C: EVALUATION OF FEEDING FREQUENCY ON THE GROWTH OF MONOLAYER EXPANDED CRYOPRESERVED CLONAL BOVINE ARTICULAR CHONDROCYTES

The literature contains varied accounts of nutritional regimes for expanding chondrocytes as highlighted in chapter A (pages 30-43). Understanding and identifying a preferred regime could be of high value to industry and healthcare outfits that require large volumes of cells. In order to evaluate the effect of feeding frequency a cell bank of clonal bovine chondrocytes was first established.

1.0 Isolation of Clonal Articular Chondrocytes, Cryopreservation and creation of the Cell Bank

Bovine chondrocytes were isolated from the Metacarpophalangeal joints of eighteen to twenty-four-month-old cows. Clonal strains were isolated using the differential adhesion to Fibronectin method described by Dowthwaite et al. (2002). Colonies that had reached approximately two hundred cells in size after five days (Fig.1) were isolated using cloning disks and transferred to T25s and allowed to become confluent before transferring to T75s. Cells were banked at P2 in DMSO-FBS and stored in vapour phase liquid nitrogen ready for subsequent experiments. In order to minimise biological variation, all experiments were performed with clonal strains obtained from a single donor unless otherwise stated.
2.0 Cell Counting Methods

Cell counting is staple of the project, and as a number of different methods were available, a brief experiment was run in order to choose the most appropriate method. A 10mL cell suspension was divided into 100µl aliquots and the aliquots randomised. Batches of aliquots were assigned to one of four methods: Manuel Haemocytometer count, Automated NC-3000 Via-1 Count, Automated NC-3000 A2 Count and Automated NC-3000 A8 count. Each count method used ten aliquots to generate ten cell counts (Fig. 2). The experimenter produced single outliers in both the Via-1 and A2 groups. In the instance of the Via-1 group the outlier corresponded to a small air bubble introduced into the cassette during sample acquisition. The same explanation does not apply to the A2 group and instead, it was postulated that the outlier was simply the result of pipetting error. In either case, the outliers highlight the importance of care in the sample preparation stage. It was decided not to remove the outliers from the data set. Off the back of the experiment, it was decided that the Via-1

![Fig.1 Images of colonies after six days of expansion. Cells were seeded at a density of approximately 104 cells/cm² on tissue culture plastic pre coated with 1µg/cm³ of bovine fibronectin. Scale bars: 100µm.]
Cassette would be the exclusive counting method used for all experiments based on a combination of ease of use, time to prepare the count and precision of the test data.

3.0 Effect of Feeding Frequency on Growth Kinetics.

Different feed intervals were imposed during a five-day expansion of the clonal chondrocyte strains to understand the impact on the growth kinetics and metabolic activity. Batch, seventy-two-hour and a twenty-four-hour feed regime were studied separately in T-flasks. The aim is to understand if increased nutritional supplementation is an effective method of increasing biomass while maintaining cell phenotype.

For each of the feeding regimes under investigation, three vials of cryopreserved clonal chondrocyte strains were thawed and expanded with serum containing medium in separate T175s for five days to reach appropriate numbers for a growth curve experiment. T25 flasks were seeded at a density of 3000 cells cm\(^2\) and supplied with 0.2mL/cm\(^2\) of FBS.
containing expansion medium and placed in a humidified incubator (37°C) with a 5% CO₂ atmosphere. The growth curve was obtained by sacrificial cell counts at twenty-four-hour intervals. Medium samples were taken from the sacrificed flasks and stored at -20°C prior to metabolite analysis. Flasks from each group were fed according to their group. Flasks belonging to the batch group were not replenished over the five-day period. Flasks from the seventy-two hour were replenished by means of a total medium exchange after seventy-two hours in culture and similarly, flasks in the twenty-four-hour feed group were replenished with medium daily. Cumulative population doublings were determined from sacrificial cell counts taken at twenty-four-hour intervals and the specific growth rates are reported for the batch (Fig.3), seventy-two hour (Fig. 4 and 5) and the twenty-four hour (Fig. 6) experiments. The mean differences of the growth rates obtained for the batch, seventy-two hour and twenty-four-hour groups were tested for significance (Fig 7).

**Fig.3** Cumulative population doublings (A) of three clonal strains over a hundred and twenty hours under batch operation. Specific growth rate (B) for the same three clonal strains over the one hundred and twenty hour period. Error bars show standard deviation of the mean (n=3)
Cumulative population doublings of the clonal strains in the batch experiment reached 2.53 ± 0.19, 2.32 ± 0.06 and 3.25 ± 0.48, respectively, after five days in culture (Fig. 3 A). The increase in biomass was equivalent to growth rates of 0.44 day⁻¹ ± 0.03, 0.40 day⁻¹ ± 0.010 and 0.50 ± day⁻¹ 0.08 for each of the strains (Fig. 3 B).

**Fig. 4** Cumulative population doublings of three clonal strains from the same donor (A) over a hundred and twenty hours under three day feed operation. Cumulative population doublings (B) of three clonal strains over a hundred and twenty hours under three day feed operation. The clonal strains used here were obtained from a different biological donor to provide a rough assessment of the impact of donor variation. Error bars show standard deviation of the mean (n=3)
Cumulative population doublings achieved for the clonal strains in the seventy-two-hour feed experiment reached 2.53 ± 0.19, 2.32 ± 0.06 and 3.25 ± 0.48, respectively, after five days in culture (Fig. 4 A). Strains 1-3 were obtained from the primary cell bank whilst strains 4-6 were obtained from a different biological donor in (Fig. 4 B). Population doublings obtained from these clonal strains reached 3.29 ± 0.47, 3.65 ± 0.34 and 3.56 ± 0.45, respectively. The corresponding growth rates for the six strains were recorded as 0.63 day$^{-1}$ ± 0.15, 0.51 day$^{-1}$ ± 0.03, 0.81 day$^{-1}$ ± 0.13, 0.63 day$^{-1}$ ± 0.06, 0.62 day$^{-1}$ ± 0.08 and 0.48 day$^{-1}$ ± 0.031. The differences in growth rates between the strains from obtained from different donors was approximately 12%.

Fig. 5 Specific growth rate for the clonal strains, combining the data from two separate biological donors onto the same axis with strains four to six from a separate donor. The growth rates were obtained from strains fed under seventy-two hour operation cultured over a five-day period. Error bars show standard deviation of the mean (n=3).
Cumulative population doublings for the three clonal strains from the twenty-four-hour feed experiment were 4.20 ± 0.06, 4.067 ± 0.27 & 3.93 ± 0.20, respectively, after five days in culture (Fig. 6 A). The increase in biomass was equivalent to growth rates 1.33 day$^{-1}$ ± 0.12, 0.75 day$^{-1}$ ± 0.21 and 0.91 day$^{-1}$ ± 0.21, respectively for each of the strains (Fig. 6 B).

**Fig.6** Cumulative population doublings (A) of three clonal strains over a hundred and twenty hours under twenty-four hour feed operation and corresponding specific growth rates (B). Error bars show standard deviation of the mean (n=3)
The difference between the growth rates from the three feed experiments were tested for significance (Fig. 7). The three data sets were judged to approximate normality before performing a one-way ANOVA. The ANOVA suggested there difference between the groups was significant with the significance threshold set at 0.05. Group data sets were tested for equality of variance before performing post-hoc T testing to ascertain between which groups a significant difference could be recorded. Three comparisons were made and as a precaution against type I error a family-wise error correction was applied in the form of the Bonferroni correction. Accordingly, the threshold for significance was set at 0.017. There was no significant difference between the means of the batch (M=0.47, SD=0.084) and seventy-two-hour feed (M=0.63, SD=0.09) conditions; t (7) 3.56, p=0.045. A significant difference between batch (M=0.47, SD=0.086) and twenty-four-hour feed (M=0.75, SD=0.11) conditions; t (16) 6.06, p=0.010 was recorded. The difference between the means
of the seventy-two-hour feed experiment and the twenty-four-hour experiment were not found to be significant, conditions; t (7) 1.82, p=0.11.

### 3.1 Discussion of Batch, Seventy-Two and Twenty-Four Hour Feed Experiments

Imposing different feeding routines on the clonal strains had a quantifiable effect on the growth kinetics of the clonal strains. The differences in specific growth rate between the twenty-four-hour feed operation for the combined clonal replicates were found to be statistically significant when compared to the batch operation (Fig.7). The difference between the seventy-two hour and the twenty-four-hour feed operation and the batch operation was not found to be significant when the Bonferroni correction was applied. The shape of the growth curves from the three experiments yields illustrates the effects of imposing different feed operations. The average number of population doublings at the seventy-two-hour time point was similar for all feed operations: 2.26, 2.42 and 2.22 for the batch, seventy-two hour and twenty-four-hour operations, respectively. After seventy-two hours the clonal strains grown under the batch feed operation began to slow and resulted in a plateau over the next forty-eight hours in two of the three clonal replicates. In contrast to this, a medium exchange at seventy-two hours sustained proliferation although appeared to induce a lag in growth in some of the clonal strains. A daily feed operation resulted in a more linear-like growth phase after seventy-two hours had elapsed.

The feed experiments suggested that a rate-limiting event is occurring around the seventy-two-hour time point that can be circumnavigated partially by re-feeding at seventy-two hours or entirely by refeeding every twenty-four hours. These findings are entirely consistent with the literature (J Melero-Martin, 2004) which reported frequent split feeds result in an enhanced growth rate of clonal chondrocytes. The same study showed that the
growth rate was serum dependent. To investigate whether the nature of the rate-limiting event is due to depletion of medium components, build-up of growth inhibiting metabolites or a combination of both. Analysis of the metabolite concentration in the growth medium was undertaken in an attempt to better define the nature of the rate-limiting event.

4.0 Metabolite Analysis of Culture Medium taken from Batch, Seventy-Two Hour and Twenty Four-Hour Feed Operation.

Frozen medium samples were thawed at room temperature and screened for glucose, glutamine, ammonia, and lactate concentration. Metabolite concentrations were determined with a Cedex Biostation HT which automates the glucose hexokinase, glutaminase, glutamate dehydrogenase and lactate oxidase assays. Assays were run as single reactions. Gross and total consumption/production are reported along with specific consumption/production rates for each of the metabolites.
Fig. 8 Gross consumption/production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the batch experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Error bars show standard deviation of the mean (n=3)
Fig. 9 Total consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the batch fed experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Lactate and ammonia concentrations were adjusted by subtracting background concentrations in the medium. Error bars show standard deviation of the mean (n=3)
**Fig. 10** Specific consumption / production rates in pmol.cell$^{-1}$ day$^{-1}$ for glucose (A), lactate (B), glutamine (C) and ammonia (D) from the batch fed experiment over a one hundred and twenty hour culture. Error bars show standard deviation of the mean (n=3)
4.1 Discussion of Batch Metabolite Data

The metabolite analysis of the batch experiment revealed some interesting points. Over the one hundred and twenty hours of culture, glucose and glutamine levels fail to deplete (Fig. 8 A and C) so cannot be the rate-limiting substrates. Over the culture period, the clonal strains consumed an average of 0.9mM and 0.98mM for glucose and glutamine, respectively (Fig. 9 A and C) This translates to an average specific consumption of 313 and 405 pmol.cell\(^{-1}\).day.\(^{-1}\) for glucose and glutamine, respectively (Fig. 10 A and C). These figures are in the range of thirty to forty times higher than those previously reported for human mesenchymal stem cells (Heathmen et al., 2015) although these bovine clonal chondrocytes display growth rates approximately 2.5 larger over a shorter time period and restricted feeding.

The accumulation of waste product is another possible origin of the stunted growth rate seen when imposing a batch operation. Typically regarded as a waste product in cell culture, lactate is produced as a by-product of glycolysis when insufficient oxygen is present to allow oxidation of pyruvate to acetyl CoA. Instead of being utilised in the tricarboxylic acid cycle the pyruvate is reversibly, converted to lactate by lactate dehydrogenase. Some cell lines can convert lactate to glucose via the Cori cycle (C Cori 1931), although, it is not known if chondrocytes have this capability. The accumulation of lactate in culture medium is known to inhibit cell growth, however, concentrations that are deemed inhibitory to growth appear to vary greatly depending on cell type. Srček et al. 2010, reported decline in growth rates of channel catfish ovary cells starting at lactate concentrations of 2.5mM, in contrast, Hayter et al., 1991 reported lactate concentrations as high as 22mM are tolerated by CHO cells. The only study to investigate the effect of lactate concentration on growth rate in chondrocytes demonstrated that growth did not plateau until the cumulative concentration reached approximately 22mM (J Malero-Martin, 2004). Two of the three clonal strains showed
average lactate concentrations in the region of 2.5 mM and the third strain, approximately
1.4 mM of lactate (Fig 9 B). With this in mind, it is unlikely that the lactate concentrations
encountered over this short culture duration were rate-limiting. While the primary aim of this
study was to identify potentially rate-limiting events and interesting metabolic behavioural
pattern was uncovered. Specific lactate production versus glucose consumption ratios were as
high as 4:1 which suggests anaerobic glycolysis dominates (Fig. 10 A and B). This apparent
behaviour arises even without imposing anaerobic conditions on the culture. This behaviour
is indicative of the Warburg effect, which describes the phenomenon of enhanced lactate
production even with nominal oxygen concentrations. This effect is associated with altered
glycolytic pathways, which in turn, is associated with some cancers (J W Locasale et a.,
2016). The significance of this observed effect in this instance is unknown however, one
concern is that the clones may have developed chromosomal abnormalities. Chromosomes 17
and 18 carry the P53 tumour suppressor gene and DCC gene. Alteration or deletion of these
genes is implicated in some colorectal cancers (J Garcia et al., 2003). Karyotypic analysis of
human clonal chondrocytes isolated using the same methodology described here have
previously shown abnormalities in the form of deletion of the long arm of chromosome 20 (R
Williams et al. 2010). The existence of karyotypic abnormalities in these strains was not
investigated further but sets a precedent for future investigation.

Ammonia is another well-documented rate limiting metabolite that is produced as a
by-product of amino acid metabolism. Ammonia has been reported, in some cell lines, to be a
more potent inhibitor of growth when compared to lactate, with concentrations as little as
1 mM having a quantifiable impact on specific growth rate (Srček et al., 2010). The average
net production of ammonia in the batch experiment was approximately 0.5 mM, so it is
unlikely that ammonia accumulation is having a significant impact on the growth rate in this
instance. While overall ammonia concentrations in the growth media were modest the
specific production were comparatively high, averaging approximately 700 pmol.cell\(^{-1}\).day\(^{-1}\) (Fig. 10 D). This observation may be linked to the apparent metabolic preference for lactate production over the oxidative phosphorylation of pyruvate. In order to help meet cellular energy requirements a higher rate of glutamine is utilised as a precursor to \(\alpha\)-ketoglutarate, an intermediary in the TCA cycle. As a consequence of over-reliance on this pathway, increased levels of ammonia are encountered as a by-product of the oxidative deamination of glutamate (Grabowska et al., 2015). Whilst re-prioritising of metabolic pathways and substrate preferences provides a sensible explanation of the high specific ammonia production rates the hypothesis is, ultimately, confounded by the non-enzymatic decomposition of glutamine in the culture medium. This decomposition obscures both the glutamine consumption rate and the ammonia production rate, with one study reporting that the difference between apparent and actual uptake/production to be in the region of 200% (O. Palsson et al., 1990). Comparative differences in seventy-two hour and twenty-four-hour feed operations may reveal if the high specific ammonia production is a common feature of these cell strains or a result of imposing a batch strategy and hence were studied next.
Fig. 11 Gross consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the seventy-two hour feed (100% exchange) experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Only clonal strains 1-3 were tracked at each twenty-four-hour time point. Error bars show standard deviation of the mean (n=3).
Fig. 12 Total consumption / production in mM/L of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the seventy-two hour feed (100% exchange) experiment over a one hundred and twenty hour culture. Offline measurements were made every twenty-four hours for replicates 1-3 and at 72h and 120h for strains 4-6. Total consumptions were calculated by the addition of the background correct values recorded at 72 and 120hs. Error bars show standard deviation of the mean (n=3)
Fig. 13 Specific consumption / production rates in pmol.cell\(^{-1}\)day\(^{-1}\) for glucose (A), lactate (B), glutamine (C) and ammonia (D) from the seventy two hour feed experiment over a one hundred and twenty hour culture. Error bars show standard deviation of the mean (n=3)
4.2 Discussion of Metabolite Date from Seventy-Two Hour Feed Operation

Metabolic behaviour in the seventy-two-hour feed experiment shows some similarities to that of the batch experiment but also some subtle differences. The average total level of glucose consumption over the culture period for all six strains was 1.13 mM (Fig. 12 (A)) which was only slightly elevated from the batch experiment but equates to a large difference in specific glucose uptake (Fig. 13 (A)). It appears that less glucose uptake, in this instance, translates to an increased growth rate and it is tempting to speculate that this could be due a shift in metabolic pathway preference that favours more efficient glucose usage. The specific lactate production was also, on average, lower with 286 pmol.cell\(^{-1}.\text{day.}^{-1}\) (Fig. 13 (B)) versus 844 pmol.cell\(^{-1}.\text{day.}^{-1}\) for the batch experiment (Fig. 10 (B)). Specific glutamine consumption was substantially lower in the seventy-two-hour feed (Fig. 13 (C)), which, on average, led to a drop in specific consumption of 310 pmol.cell\(^{-1}.\text{day.}^{-1}\) over the batch culture (Fig. 10 (C)). Related to this was a large drop in specific ammonia production (Fig. 13 (D)) compared to the batch experiment (Fig. 10 (D)). The specific lactate, glutamine and ammonia fluxes are all consistent with the view of a shift in metabolic pathway preference that favours the oxidative phosphorylation of pyruvate with less demand on glutamine as an alternate energy source.

Although specific lactate and ammonia fluxes are lower than those observed in the batch experiment, absolute levels were higher. Gross lactate and ammonia concentrations both averaged in the region of 2.3mM. Interestingly, maximum ammonia and lactate concentrations of 1.1mM and 2.5mM, respectively, were experienced at seventy-two hours but growth for all of the clonal replicates endured. This suggests that lactate and ammonia concentrations in this region are, to some extent, tolerated by this cell type.
Fig. 14 Gross consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the seventy-two hour feed (100% exchange) experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Only clonal strains 1-3 were tracked at each twenty-four-hour time point. Error bars show standard deviation of the mean (n=3).
Fig. 15 Total consumption/production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the twenty-four hour feed (100% exchange) experiment over a one hundred and twenty hour culture. Total consumptions were calculated by the addition of the background correct totals recorded at each twenty-four-hour interval. Error bars show standard deviation of the mean (n=3)
Fig. 16 Specific consumption / production rates in pmol.cell$^{-1}$.day$^{-1}$ for glucose (A), lactate (B), glutamine (C) and ammonia (D) from the twenty four hour feed experiment over a one hundred and twenty hour culture. Error bars show standard deviation of the mean (n=3)
4.3 Discussion of Metabolite Date from Twenty-Four Hour Feed Operation

Data from the twenty-four-hour feed experiment suggest that the imposition of this frequency of feeding promotes more efficient use of glucose with respect to specific consumption when compared to batch and seventy-two-hour feed modes. Glucose to lactate ratios where similar in the twenty-four-hour experiment neared a 1:1 consumption to production ratio (Fig 16 (A) and (B)). Total lactate and ammonia (Fig 15 (B) and (D)) accumulation was minimal and thought unlikely to impede growth kinetics. A general observation was noted that the metabolic behaviour of the cell strains was rather homogenous in the twenty-four-hour feed experiment when compared to the data sets from the batch and seventy-two-hour feed experiments. The significance of this observation or its direct implication on cell expansion strategy is not currently known.

4.4 Summary of Metabolite Results from Batch, Seventy-Two-Hour and Twenty-Four-Hour Feed Experiments.

The metabolite data obtained from the three experiments allows a number of observations to be drawn. The differences in growth rate of the clonal strains observed because of the three feeding regimes:

- Was not caused by depletion of glucose or glutamine.
- Maybe partly accounted for by build-up of lactate and/or ammonia. Although the total differences in metabolite consumption or production between 24h, 72 and fed-batch regimes were not statistically significant (Fig. 17)
Maybe dose dependent behavior to components contained within the Fetal Calf Serum.

Publications to date have demonstrated the growth kinetics can be enhanced in a dose-dependent manner by supplementing the growth medium with recombinant proteins such as fibroblastic growth factor (K H Chua et al., 2007., Gerjo van Osch et al., 2002. ) and Platelet Derived Growth Factor-BB (J Wroblewski and C Edwall, 1992) as well as serum on its own (Chua KH, 2004).

Mammalian cell culture is hugely dependent on the use of fetal calf serum. The exact composition of serum varies from batch to batch but can be summarized as a mixture rich in albumin, insulin, cortisone, thyroxine, PTH (Parathyroid Hormone), PGE (Prostaglandin E),

![Comparison of differences in metabolite consumption or production from the 24h, 72h and fed batch experiments. The differences between the groups were tested for significance using ANOVA. No differences were found to be significant. Error bars show standard deviation of the mean (n=3).](image)
Cholesterol and fatty acids (P J Price & E A Gregory, 1982). Serum use is widespread owing to the fact it is readily available, relatively affordable and supports the growth of a diverse range of cell lines. Despite these apparent advantages of serum, there are number of arguments against the use of serum. Serum by nature is a poorly defined component of cell culture and varies from batch to batch. The manufacture of therapeutic cells will likely follow a similar regulatory framework to that that overseas the manufacturer of pharmaceuticals. This demands that the operating space be under tight control of the user, with all variables that can affect quality accounted for (FDA Guidance for Industry, 2009). Further, some of the components of serum may induce undesirable phenotypic changes in the cell line during culture. Any large-scale cell therapy that may employ the use of human serum will be wary of the possibility of adventitious agents that may be present in the natural product. A large demand for serum may also place pressure on blood banks sparking supply chain problems and spark an ethical debate on the use of donated whole blood (Zielinski MD et al., 2014). Finally, there is an ethical argument to be made to reduce the reliance on fetal calf serum in mammalian cell culture in a bid to reduce the burden on animals and align research practice with the 3Rs.

A chemically defined medium substitutes serum for known quantities of naturally sourced or recombinant proteins. The exact composition is specific to the cell type. A number of publications to date have published a “recipe” for a chemically defined medium that supports the growth of human chondrocytes (Xin-xin Shao et al., 2013., P. Giannoni et al. 2005., Malpeli et al., 2004). While the formulas vary from author, they all share some common components, namely albumin, ITS, EGF, FGF-2 and PDGF-BB. These formulations formed the basis for the defined medium described herein. The objective of using a defined medium is to ultimately, understand exactly how the individual components influence growth.
rate, metabolic behaviour, and phenotype. The initial studies aim to replicate the work completed using the serum-containing medium with a defined medium.
5.0 Evaluation of Feeding Frequency on the growth of monolayer-expanded cryo preserved clonal bovine articular chondrocytes using a chemically defined medium

5.1 Formulation of a Chemically Defined Medium

A chemically defined medium was developed and tested to assess its impact on cells cryopreserved in serum. The composition of the medium is detailed in table 1. In the absence of serum, an appropriate adhesion protein is required. Fibronectin was selected due to the fact that the initial cell population was selected based off an affinity for the protein. Initial experiments (data not shown) resulted in complete loss of the culture after twenty-four hours. Further investigation revealed that the pH of the formulation increased to as high as 8.4 during manufacture possibly because of degassing. This behaviour was not observed with the serum-containing medium (SCM). However, the SCM was double buffered with HEPES rendering the pH CO₂ independent. The pH fluctuations were addressed first by introducing HEPES into the culture. Addition of HEPES to the medium did help attenuate the pH and allowed for a small degree of proliferation. Morphological assessment of the cells revealed the presence of HEPES was inducing cytoplasmic vacuolation and an overall shrivelled appearance (Fig.18). The addition of HEPES also increased the osmolarity to the 350 mOsmol/kg range which appeared to be poorly tolerated. In light of this, the incorporation of HEPES into the medium was deemed an unsatisfactory solution. Titration of the medium with 1N HCl, although initially thought of as a poor choice, was the most effective at reducing the pH to about 7.3-7.4 range while maintaining sterility. Once the pH was lowered and placed into the culture vessel 5% CO₂ appeared adequate at maintaining the pH. Sparging the stock medium with CO₂ may be a preferable alternative to HCl titration, however,
equipment limitations made achieving this in an aseptic manner was problematic. Once the pH problem was overcome it was found that medium could support the proliferation of the clonal chondrocytes (Chapter B, 1.8). It was found that serum weaning or adaption passages were not required to support a base bulk growth of the clonal lines, which are necessary for some cell lines (J van der Valk et al., 2010). Cell subpopulations within the clonal lines were not characterised and monitored so the possibility that the defined medium was introducing pressure selection cannot be ruled out and remains an unknown. Understanding pressure selection presents itself as a priority area for future work in relation to the commercialization of cell lines as the uncertainty could undermine the principle of quality.
Table.1 Chemically defined medium. Volumes of 1N HCl added varied but were typically in the range of 250-300 µl per 100mL of medium. Quoted volumes of recombinant proteins and dexamethasone refer to the stored aliquots.

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Component Storage</th>
<th>Concentration in final formulation</th>
<th>Weight / Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Thermo Fischer Scientific</td>
<td>A1443001</td>
<td>RT</td>
<td>N/A</td>
<td>94.95mL</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>Sigma Aldrich</td>
<td>G7021-1KG</td>
<td>RT</td>
<td>1g/L</td>
<td>100mg</td>
</tr>
<tr>
<td>Bovine Serum Albumin (Fraction V)</td>
<td>Sigma Aldrich</td>
<td>A9418-10G</td>
<td>2-8°C</td>
<td>1% w/v</td>
<td>1g</td>
</tr>
<tr>
<td>Chemically Defined Lipid Mixture 1</td>
<td>Sigma Aldrich</td>
<td>L0288-100ML</td>
<td>2-8°C</td>
<td>0.5% v/v</td>
<td>0.5mL</td>
</tr>
<tr>
<td>Kao and Michayluk Vitamin Solution (100x)</td>
<td>Sigma Aldrich</td>
<td>K3129-100ML</td>
<td>-20°C</td>
<td>1% v/v</td>
<td>1mL</td>
</tr>
<tr>
<td>L-Glutamine (200mM)</td>
<td>Thermo Fischer Scientific</td>
<td>25030081</td>
<td>2-8°C</td>
<td>5mM</td>
<td>2.5mL</td>
</tr>
<tr>
<td>ITS (100x)</td>
<td>Sigma Aldrich</td>
<td>I2521-5ML</td>
<td>2-8°C</td>
<td>1% v/v</td>
<td>1mL</td>
</tr>
<tr>
<td>Dexamethasone‡</td>
<td>Sigma Aldrich</td>
<td>D4902-100MG</td>
<td>-20°C</td>
<td>10⁻⁴ M mol/L</td>
<td>40µL</td>
</tr>
<tr>
<td>Human Recombinant PDGF-BB‡</td>
<td>R&amp;D Systems</td>
<td>220-BB-010</td>
<td>-80°C</td>
<td>10ng/mL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Human Recombinant FGF²</td>
<td>R&amp;D Systems</td>
<td>233-FB-025/CF</td>
<td>-80°C</td>
<td>10ng/mL</td>
<td>8 µL</td>
</tr>
<tr>
<td>Human Recombinant EGF‡</td>
<td>R&amp;D Systems</td>
<td>236-EG-200</td>
<td>-80°C</td>
<td>10ng/mL</td>
<td>50 µL</td>
</tr>
<tr>
<td>HCl (1N)</td>
<td>Sigma Aldrich</td>
<td>H9892-100ML</td>
<td>RT</td>
<td>Variable</td>
<td>Variable</td>
</tr>
</tbody>
</table>
5.2 Effect of Feeding Frequency on Growth Kinetics.

The impact of batch feeding, seventy-two hour total medium exchanges and twenty-four-hour total exchanges were studied in three independent experiments using cryopreserved clonal chondrocytes and conventional tissue culture plastic in a manner identical to the serum-based experiments. The clonal strains used in this section were from the same donor as in the previous. The aim is to understand if increased nutritional supplementation is effective at enhancing proliferation like the serum-containing medium was.

For each of the feeding regimes under investigation three vials of cryopreserved clonal chondrocytes were thawed and expanded with the chemically defined medium in separate T175s for five days to reach appropriate numbers for a growth curve experiment. The T175 flasks were precoated with 1μg/cm³ of bovine fibronectin and left in an incubator at 37°C for two hours prior to inoculation. Six-well plates were used instead of T25s to make the experiments more cost-effective. Well plates were again precoated with 1μg/cm³ of fibronectin and cells were seeded at a density of 3000 cells cm², supplied with 0.2mL/cm² of defined medium and placed in a humidified incubator (37°C) with a 5% CO₂ atmosphere. The

Fig.18 Image of clonal chondrocytes displaying HEPES induces stress. Scale bar: 20μm
growth curve was obtained by sacrificial cell counts at twenty-four-hour intervals. Medium samples were taken from the sacrificed flasks and stored at -20°C prior to metabolite analysis. Flasks from each group were fed according to their group like the serum-based experiments.

**Fig. 19 (A)** Cumulative population doublings of three clonal strains over a hundred and twenty hours under batch fed operation. **(B)** Specific growth rate for the same three clonal strains over the one hundred and twenty hour period. Error bars show standard deviation of the mean (n=3)

Averaged cumulative population doublings of the clonal strains from the batch experiment reached 0.99 ± 0.49, 1.19 ±0.27 and 0.94 ± 0.29, respectively, after five days in culture (Fig. 19 (A)). The increase in biomass was equivalent to averaged specific growth rates of 0.17 day⁻¹ ± 0.09, 0.14 day⁻¹ ± 0.05 and 0.20 day⁻¹ ± 0.02 for each of the strains, respectively (Fig. 19 (B)).
Averaged cumulative population doublings of the clonal strains from the seventy-two-hour feed experiment reached 2.65 ± 0.61, 2.67 ± 0.24 & 3.08 ± 0.28, respectively, after five days in culture (Fig. 20 (A)). The increase in biomass was equivalent to averaged specific growth rates of 0.47 day\(^{-1}\) ± 0.07, 0.46 day\(^{-1}\) ± 0.04 & 0.53 day\(^{-1}\) ± 0.05 for each of the strains, respectively (Fig. 20 (B)).
Averaged cumulative population doublings of the clonal strains from the twenty-four-hour feed experiment reached 3.49 ± 0.54, 3.42 ± 0.37 & 3.56 ± 0.49, respectively, after five days in culture (Fig. 21 (A)). The increase in biomass was equivalent to averaged specific growth rates of 0.60 day$^{-1}$ ± 0.09, 0.59 day$^{-1}$ ± 0.06 & 0.62 day$^{-1}$ ± 0.08 for each of the strains, respectively (Fig. 21 (B)).

**Fig. 21 (A)** Cumulative population doublings of three clonal strains over a hundred and twenty hours under twenty-four hour feed operation. The three strains reached average cumulative doublings of 3.49 ± 0.54, 3.42 ± 0.37 & 3.56 ± 0.49, respectively. **(B)** Specific growth rate for the same three clonal strains over the one hundred and twenty hour period. Under seventy two feed operation, rates of 0.60 day$^{-1}$ ± 0.09, 0.59 day$^{-1}$ ± 0.06 & 0.62 day$^{-1}$ ± 0.08 were achieved for the three clonal strains, respectively. Error bars show standard deviation of the mean (n=3).
The difference between the growth rates from the three feed experiments were tested for significance. The three data sets were judged to approximate normality before performing a one-way ANOVA. The ANOVA suggested the difference between the groups was significant with the significance threshold set at 0.05. Group data sets were tested for equality of variance before performing post-hoc T testing to ascertain between which groups a significant difference could be recorded. Three comparisons were made and as a precaution against type I error a family-wise error correction was applied in the form of the Bonferroni correction. Accordingly, the threshold for significance was set at 0.017. There was a significant difference between the means of the batch (M=0.17, SD=0.034) and seventy-two hour feed (M=0.45, SD=0.08) conditions; \(t(4) = 5.1, p=0.0069\), and the batch (M=0.17, SD=0.034) and twenty-four-hour feed (M=0.60, SD=0.026) conditions; \(t(4) = 2.76, p=6.75E^{-5}\).

The difference between the means of the seventy-two-hour feed experiment and the twenty-

**Fig.22 (A)** Averaged Specific growth rates for the all clonal strains across the three different feed operations over the one hundred and twenty hour period. Significance was tested using a single factor ANOVA (\(\alpha=0.05\)) which yielded a \(p\) value of 0.00026. Individual significances were calculated using Bonferroni corrected post-hoc T tests. Error bars show standard deviation (n=6). **(B)** Averaged specific rates for all clonal strains comparing rates obtained from the serum-containing medium and those obtained using the chemically defined medium. Error bars show standard deviation of the mean (n=3).
four-hour experiment were not found to be significant (p=0.05) after the error correction was applied.

5.3 Discussion of Batch, Seventy-two hour and Twenty-four-hour feed Operations using the Chemically Defined Medium.

The chemically defined medium enabled proliferation of the clonal chondrocytes in a manner comparable to what was achieved using a serum-based medium. Like in the serum-based experiments, specific growth rates increased by statistically significant margins as the frequency of feeding was increased (Fig. 21 (A)). The batch culture resulted in the biomass reaching a peak at around seventy-two hours in two of the clonal strains, with the third strain reaching a peak at ninety-six hours before declining (Fig. 18 (A)). The seventy-two-hour feed experiments resulted in a near inflection of growth after only forty-eight hours (Fig. 19 (A)). The feeding intervention at seventy-two hours allowed proliferation to resume in all of the clonal strains, albeit to varying extents. The twenty-four-hour feed frequency resulted in the highest growth rates and equated to a respective average increase of 74% and 25% relative to the batch fed and seventy-two-hour feed (Fig. 20 (A)). Comparing the serum to the chemically defined medium reveals the serum-containing medium is superior, at least in terms of stimulating proliferation. This result is perhaps not unexpected given the chemically defined medium is suboptimal and is susceptible to pH fluctuations. Consideration also has to be given to the fact that the colonies were isolated, expanded and cryopreserved with serum-containing medium. It has been reported in the literature that switching a SCM for a defined medium requires a “weaning period” or a stepwise reduction of serum concentration in order to support survival and growth (JBF van der Valk, 2010). While it was found an adaption
passages were not required for cell survival and growth, the possibility that abruptly removing serum from the medium could affect the growth rate cannot be discounted. Understanding what is directly responsible for stimulating proliferation is key to developing a tailored, programmable approach to nutrition. To be pragmatic in terms of approach, the same metabolite screening will be employed in a bid to uncover any rate limiting events and understand any general trends in metabolic behaviour.

6.0 Metabolite Analysis of Culture Medium taken from Batch, Seventy-Two Hour and Twenty Four-Hour Feed Experiments Cultured using a Chemically Defined Medium.

Frozen medium samples were thawed at room temperature and screened for glucose, glutamine, ammonia, and lactate concentration. Metabolite concentrations were determined with a Cedex Biostation HT which automates the glucose hexokinase, glutaminase, glutamate dehydrogenase and lactate oxidase assays. Assays were run as single reactions. Cell normalised consumption or production rates were calculated using the same expression as described in section 2.4
Fig. 23 Gross consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the fed batch experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Error bars show standard deviation of the mean (n=3)
Fig. 24 Total consumption/production in mM of glucose(A), lactate (B), glutamine (C) and ammonia(D) from the fed batch experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Lactate and ammonia concentrations were adjusted by subtracting background concentrations in the medium. Error bars show standard deviation of the mean (n=3).
**Fig. 25** Specific consumption / production rates in pmol.cell$^{-1}$.day$^{-1}$ for glucose(A), lactate (B), glutamine (C) and ammonia(D) from the batch experiment over a one hundred and twenty hour culture. Error bars show standard deviation of the mean (n=3)
The metabolite data from the batch experiment using the chemically defined medium revealed a higher specific glucose consumption rate to that of the serum experiment (Fig. 24 (A) and (B)). Glucose to lactate ratios were roughly 1:2 which although lower than those encountered in the serum experiment still suggests inefficient glucose usage. Absolute levels of glucose in the defined medium were approximately a third lower to those in the serum (Fig. 24 (A) and did not come close to depleting over the course of the culture, which at the very least suggests high-glucose formulations are wasteful. High glucose formulations have been demonstrated to be detrimental in some cell types (A Stolzing, 2006., Yu-Cheng Lin et al. 2017 ) however the data produced from the serum containing experiments, which contained approximately three times as much glucose as the defined medium, showed superior growth rates versus the lower glucose defined medium. This observation could arise from a number of scenarios such as, additional mitogenic components present in the serum compensating for any potential rate-limiting effects that exposure to high glucose may incur, the relatively short culture periods masking any effect or the cell strain being insensitive to glucose concentration. Glutamine and ammonia levels recorded from the experiment were confounding (Fig.23 (C) and (D)). Glutamine levels rose significantly at seventy-two hours without any intervention in all three of the clonal lines, as did ammonia levels fall in two of the clonal lines at the same time point. Note that no such fluctuation is experienced with the recorded glucose and lactate levels (Fig. 23 (A). Although the glutamine and ammonia fluctuations could be rationalised in terms of cell biology it is more likely the results arose from either a problem with assay calibration or sample contamination. Data from the seventy-two and twenty-four-hour feed experiments will help reveal if this is a feature of using a defined medium or a random error.
Fig. 26 Gross consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the seventy-two hour feed experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Error bars show standard deviation of the mean (n=3)
Fig. 27 Total consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the seventy-two hour feed experiment over a one hundred and twenty hour culture period with offline measurements made twenty-four hours apart. Lactate and ammonia concentrations were adjusted by subtracting background concentrations in the medium. Error bars show standard deviation of the mean (n=3)
**Fig. 28** Specific consumption / production rates in pmol.cell$^{-1}$.day$^{-1}$ for glucose (A), lactate (B), glutamine (C) and ammonia (D) from the seventy-two hour experiment over a one hundred and twenty hour culture. Error bars show standard deviation of the mean (n=3).
Total glucose consumption was similar to that of the serum experiments however; total lactate production levels were far higher (Fig. 26 (D)). The determined lactate levels were less likely to affect the growth rate than the equivalent serum experiment as peak concentrations in the medium were lower due to the chemically defined medium been absent of background lactate (Fig. 25 (B)). Specific consumption rates of glucose were again far higher than the corresponding serum experiment and glucose to lactate ratios were in the region of 1:3 (Fig. 26 (A) and (B)). Specific glutamine consumption was also comparatively high suggesting a higher reliance on the amino acid to meet cellular energy requirements (Fig. 26 (C)). Ammonia levels were found to be quite variable amongst technical replicates but average peak concentrations were in the region of 1mM, which were not thought to have a significant impact on the growth kinetics.
Fig. 29 Gross consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the twenty-four hour feed experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Error bars show standard deviation of the mean (n=3)
Fig. 30 Total consumption / production in mM of glucose (A), lactate (B), glutamine (C) and ammonia (D) from the twenty-four hour feed experiment over a one hundred and twenty hour culture with offline measurements made twenty-four hours apart. Lactate and ammonia concentrations were adjusted by subtracting background concentrations in the medium. Error bars show standard deviation of the mean (n=3)
Fig. 31 Specific consumption / production rates in pmol.cell$^{-1}$.day$^{-1}$ for glucose (A), lactate (B), glutamine (C) and ammonia (D) from the twenty-four hour feed experiment over a one hundred and twenty hour culture. Error bars show standard deviation of the mean (n=3)
The determined metabolic fluxes for the twenty-four-hour experiment were in line with the previous chemically defined medium experiments. Specific glucose to lactate conversion ratios were in the region of 1:2 to 1:3 (Fig. 30 (A) and (B)), comparable to those contained in the serum-based experiments. The high levels of total lactate and ammonia concentrations (Fig. 30 (B) and (D)) were offset by the daily 100% medium exchanges and so peak levels were in line to the batch and seventy-two-hour feed experiment (Fig. 29 (B) and (D)). Glucose and glutamine levels were not exhausted during the experiment and so growth appears to be a function of one or more of the proteins in the formulation much in the same way growth appeared to be serum dependent in the initial experiments. Unlike the serum based experiments, differences in total consumption between twenty-four hour, seventy-two hour and batch fed experiments were statistically significant (Fig.32)

**Fig.32** Comparison of differences in metabolite consumption or production from the 24h, 72h and fed batch experiments using the defined medium. The differences between the groups were tested for significance using ANOVA. Groups showing significant differences were subjected to post hoc tests. The asterisk donates where significant differences after Bonferroni error correction were recorded. Errors bars show standard deviation of the mean (n=3)
Glucose consumption was significantly higher in the twenty-four hour experiments (M=1.95 SD=0.28) than the recorded consumption in the batch experiments (M=0.64 SD=0.07) conditions; t(4) 5.37 p=0.005. Glutamine consumptions were significantly higher in both the twenty-four and seventy-two hour experiments (M=1.57 SD=0.28 and M=1.29 SD=0.26, respectively) when compared to the batch experiments (M=0.25 SD=0.06) conditions; (t)4 7.88 p=0.001 and (t)4 6.71 p=0.002, respectively. Lactate production was again significantly higher in both twenty-four and seventy-two hour experiments (M=4.86 SD=0.33 and M=4.04 SD=0.40, respectively) when compared to the batch experiment (M= 1.13 SD=0.03) conditions; (t)4 14.67 p=1.25x10^{-4} and (t)4 19.43 p=2.07x10^{-5}, respectively. Ammonia production was significantly different in all three experimental scenarios. Ammonia production was higher in the twenty-four experiment (M=4.51 SD=0.09) when compared to the seventy two hour (M=2.21 SD=0.45) and the batch experiment (M=0.75 SD =0.04) conditions; (t)4 8.75 p=9.41x10^{-4} and (t)4 68.94 p=2.65x10^{-7}, respectively. The difference between the seventy-two hour and batch experiment was also found to be significant conditions; (t)4 5.67 p=0.005. Although some of the experimental scenarios resulted in marked differences in total consumption/production values it is not known if this translates into any practical significance.

The serum-based medium was slightly superior in terms of stimulating proliferation of the clonal strains, however, these experiments have yet to address the phenotypic changes that can be induced by culturing in the different mediums. An assessment of these changes needs to be undertaken before a decision can be made on which medium formulation can be taken forward for further investigation.
7.0 Quantitative PCR assessment of the impact of using serum-based medium and chemically defined medium on expression of SOX-5, 6 & 9

The SOX family of genes code for transcription factors that are ubiquitous amongst eukaryotes (P. Koopman et al. 2000). While the role of SOX proteins is varied, their role in cartilage is thought to be central in maintaining the phenotype of articular chondrocytes, in particular, SOX-5, SOX-6 and SOX-9 (E. Kolettas et al., 2001, S. Ikegawa et al., 2004). Determination of SOX-5, 6 & 9 mRNA levels, while unlikely to categorically represent quality in terms of chondrocyte phenotype, are a fair indicator of how dedifferentiated the cell population has become. SOX-5, 6 & 9 gene expression levels are the sole measure of quality in this study and if and how levels vary as a result of culture in the two different mediums is the first task. Selection of the SOX trio as a sole means of assessing quality is a weakness of the study that has been sustained due to budgetary constraints. Whilst they are strong predictors for the induction of efficient matrix producing chondrocytes it is important to acknowledge that the presence of the appropriate transcriptome does not necessarily translate into the desired proteome. Ideally, both the transcriptome and corresponding proteome would be assessed in tandem. Furthermore, other makers such as Karyotype analysis and cell surface antigen profiles could play an equally important role in product characterisation.

Six vials of cryopreserved clonal strains were thawed and expanded in individual T175’s for five days to reach appropriate cells numbers. Cells were then harvested and seeded in six-well plates at a density of 3000 cells cm$^2$. Well plates used to be used in conjunction with the chemically defined medium were pre-coated with bovine Fibronectin (1 µg/cm$^2$) prior to seeding. Each well was supplemented with either the serum-containing
medium or the chemically defined medium (0.2mL/cm²) and expanded in batch mode for a period of five days. After the five days cells were harvested and stored at -20°C in RNA cell protect ready for RNA extraction and subsequent RT-qPCR. A full account can be found in the materials and methods section.

**Fig.33** SOX-9 mRNA transcripts copies from clonal strains expanded over five days in either the serum based medium or the chemically defined medium. mRNA transcripts were determined using RT-qPCR. The differences between the serum and defined group were found to be significant ($p=0.05$) using students T test. Groups were first tested for equality of variance prior to significance testing. Error bars show standard deviation of the mean (n=3)
Fig. 34 SOX-6 mRNA transcripts copies from clonal strains expanded over five days in either the serum based medium or the chemically defined medium. mRNA transcripts were determined using RT-qPCR. The differences between the serum and defined group were not found to be significant ($p=0.05$) using students T test. Groups were first tested for equality of variance prior to significance testing. Error bars show standard deviation of the mean ($n=3$).
SOX-9 mRNA transcripts were significantly higher in all three of the strains (M=1200 SD=194, M=1069 SD=120, M=996 SD=31, respectively) in the chemically defined treatment group when compared to the strains cultured in the serum medium treatment group (M=273 SD=181, M=568 SD=107, M=664 SD=85, respectively) conditions; t(4) 6.04 p=0.004, t(3) 4.9, p=0.01, t(4) 6.3, p=0.003, respectively (Fig.31).
8.0 Summary of Chapter C Studies

Maintaining SOX-9 expression during culture is thought to be a key objective in the culture of chondrocytes as loss of SOX-9 transcripts is associated with loss of downstream extracellular matrix markers of chondrocyte phenotype collagen II and results in increased apoptosis (D. Ikegami et al., 2011, K. Von der Mark et al. 1977). SOX-6 and 5 transcript markers did not vary significantly (Fig. 32 and 33), however whether or not the two mediums do promote real differences in the expression of these transcripts is hard to say when the statistical power is low.

The experiments with the serum and chemically defined medium have revealed that the growth rate of the clonal strains is dependent on one or more of the components in the medium and that growth rate can be enhanced by increasing the frequency of feeding. Additionally, the slowing or arrest of growth is not the sole result of lactate or ammonia accumulation or depletion of glucose or glutamine.

![Graph](image.png)

**Fig. 35** A cost comparison of serum versus the chemically defined medium per litre. Costs were calculated based on current order values placed to a range of suppliers. The cost of the defined medium does not take in account pre coating tissue culture plastic with fibronectin. Costs accurate at time of composing (December 2017)
While the use of serum may appear expedient, the serum-free medium was found to be superior in maintaining SOX-9 expression and maintaining phenotypic stability is a key milestone in been able to propagate large numbers of therapeutically viable cells. This finding was consistent with other studies that experimented with expanding full thickness chondrocytes using a defined medium (D J Huey et al., 2013). A major drawback of using a chemically defined medium in this instance is the cost factor. In its current format, the chemically defined medium is approximately seven times that of the serum formulation without factoring in the adhesion protein. This currently leaves the use of a chemically defined medium an expensive proposition. In order to address this and further understand what each of the individual components in the chemically defined medium are contributing, in terms of rates of proliferation and upregulation of the SOX genes, a careful screening experiment is proposed for the next chapter.
1.0 Introduction

The defined medium described in chapter B was demonstrated to support the growth of the clonal chondrocyte cell line in a manner that was similar to the serum-containing medium with the added benefit of upregulating SOX-9 expression. In order for the proposition of a defined approach to feeding to be of optimum value, the contribution of each component to the quality attributes, growth rate and SOX gene expression in this case, needs to be carefully considered. It is envisaged that a well understood operating space will not only assist in yielding large numbers of quality cells but will lower the cost burden associated with using a defined medium by promoting efficient use of expensive recombinant proteins.

Factorial design of experiment offers an easily accessible means of screening multiple medium components for their effect on a range of independent variables (L M Collins et al., 2010). The literature presents a number of examples of this type of design applied to the field of cellular biology to identify primary and interaction effects (L Enochson et al., 2012). It was envisaged that primary effects and two-way interactions could be resolved using a fractional factorial experiment in a manner that balances the desire to uncover as much information about the system as possible with the amount of experimental resource available. Ideally, all components of the serum-containing medium and, any more of interest would be screened for their impact on the output variable of interest. In reality, only a small number of dependent variables can be screened with the resources available and so before executing the experiment candidates for screening need to be prioritised. The review of the literature
looking at commonly employed culture conditions (Chapter A) gives rise to Table 1, which explores the arguments for inclusion of a range of candidates for screening. It was decided there was enough resource to screen approximately nine candidates depending on the desired level of power.

**Table 1.** Exploration of possible candidates for screening in a fractional factorial experiment

<table>
<thead>
<tr>
<th>Candidate</th>
<th>Argument</th>
<th>Include</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Elementary media component that may exhibit main effects in conjunction with hypoxic conditions. Hyaline cartilage being avascular may benefit from low glucose concentrations or prefer different carbon sources. High glucose concentrations have been demonstrated to exhibit undesirable effects in some cell types by some investigators (A Stolzing et al., 2006., C Perio et al. 2001., H Heywood et al., 2014) but not by others (B Weil et al., 2009).</td>
<td>Yes</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>The critical medium component may be able to modulate metabolic pathway preference when presented either in excess or as a limiting factor. Investigators experimenting with human dental pulp stem cells concluded glutamine deprivation combined with low glucose was better for cell viability (N Machado et al., 2014)</td>
<td>Yes</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>An evidence base exists that this sugar effects on proliferation and gene expression in chondrocytes. The researchers reported a dose-response up to 2mM (S Varghese et al., 2007). Glucosamine will be excluded from screening because of the existing in-depth study.</td>
<td>No</td>
</tr>
<tr>
<td>Ascorbic Acid-2-Phosphate</td>
<td>Study by Enochson <em>et al.</em> (2012) indicated this did not display dose-response behaviour in three-dimension culture. Rejecting here in favour of including previously less explored options.</td>
<td>No</td>
</tr>
<tr>
<td>Serum</td>
<td>The study aims to move away from the use of undefined components.</td>
<td>No</td>
</tr>
<tr>
<td>pH / Buffer Choice</td>
<td>Undoubtedly illicit a large effect as touched on in chapter B but rejected due to technical limitations. Maintaining the pH at two distinct levels for a factorial DoE (three states with the inclusion of centre points) is experimentally demanding given the level of fine pH control that can be achieved using a standard incubator.</td>
<td>No</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Fibronectin is a constituent of fetal calf serum. It enhances cell adhesion and critically, cell spreading and cell spreading. Its impact on SOX-9 expression has previously been reported (T E Hardingham <em>et al.</em>, 2008) but less is known about its effect on SOX-6 and 5. Also, previously reported effects pertained to full thickness chondrocytes.</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Vitronectin</strong></td>
<td>Another adhesion promoter that is present in calf serum (E G Haymen, 1985). Its impact on growth kinetics and SOX transcript expression in clonal chondrocytes is unexplored.</td>
<td>No</td>
</tr>
<tr>
<td><strong>O$_2$ Tension</strong></td>
<td>Multiple studies have shown expansion under hypoxia positively influences chondrogenic potential (B D Markway et al., 2013., E Duval et al., 2009 and G Martin et al., 2004) and work demonstrating novel interaction effects with other components has yet be published. Culturing and analysis of cells under hypoxia requires appropriate workstations that are not currently available.</td>
<td>No</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>Again, undoubtedly will exhibit a main effect if test over a large enough range and may present itself as parameter for optimisation but rejecting in favour of higher priority candidates.</td>
<td>No</td>
</tr>
<tr>
<td><strong>Seed Density</strong></td>
<td>It has been suggested that high-density cultures may promote SOX-9 gene expression by limiting cell spreading. (T E Hardingham et al., 2008). Effect of low-density cultures on growth rate is also of interest.</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Transferrin</strong></td>
<td>Evidence base suggesting influential in up-regulating chondrocyte phenotype (K H Chua et al., 2005). Was included in the Enochson study, however, they reported no significant effect in redifferentiation culture. Will be included as part of a commercial formulation supplement.</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Selenium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>No evidence to suggest a role in chondrocyte phenotype modification.</td>
<td>No</td>
</tr>
<tr>
<td><strong>Biotin</strong></td>
<td>No evidence to suggest a role in chondrocyte phenotype modification.</td>
<td>No</td>
</tr>
<tr>
<td><strong>Dexamethasone</strong></td>
<td>Studies agree it has a role in down regulating proinflammatory responses (J H Jafari et al., 1993) and inducing chondrogenic differentiation (A Derfoul et al., 2006). The Enochson study (2012) confirmed it as an active variable and recommended optimal supplementation concentration.</td>
<td>No</td>
</tr>
<tr>
<td><strong>Linoleic Acid</strong></td>
<td>Linoleic acid reportedly has a pro-inflammatory effect on cartilage when in the presence of TNFα (Y.M. Bastiaansen-Jenniskens et al., 2013).</td>
<td>No</td>
</tr>
<tr>
<td><strong>Albumin</strong></td>
<td>Major protein component of serum that has a multifaceted role in cell culture (G L Francis, 2010). Not included in favour of inclusion of other components.</td>
<td>No</td>
</tr>
<tr>
<td><strong>Pyruvate</strong></td>
<td>May exhibit effects under hypoxic conditions but not practical to assess fluxes due to production from glycolysis altering the exogenous concentration by unknown amount.</td>
<td>No</td>
</tr>
<tr>
<td><strong>Pantothenate</strong></td>
<td>No evidence to suggest a role in chondrocyte phenotype modification.</td>
<td>No</td>
</tr>
<tr>
<td><strong>FGF-2</strong></td>
<td>An established evidence base for the role of FGF2 in up-regulating ear chondrocyte phenotype (E W Mandl, 2002). The growth factor has also been included in some MACT expansion regimes and has been noted to display mitogenic properties in chondrocyte culture ((D Gospodarowicz and A L Mescher, 1977). Should be included</td>
<td>Yes</td>
</tr>
</tbody>
</table>
to confirm the effect in clonal chondrocytes and to understand relationship dynamics better.

<table>
<thead>
<tr>
<th>Candidate</th>
<th>Description</th>
<th>Included for confirmation of literature reports</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-bb</td>
<td>Reported to boost proliferation and included as part of literature described total medium (Jakob, M. et al., 2001)</td>
<td>Yes</td>
</tr>
<tr>
<td>EGF</td>
<td>Reported to play a role in up-regulating chondrocyte phenotype however the study design confounds the growth factor with others, so its exact effect is not immediately apparent (APA Prins et al., 1982). Also reported to display mitogenic properties in chondrocytes (D Gospodarowicz and A L Mescher, 1977).</td>
<td>Yes</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Controversial growth factor. Numerous studies shed light on its role in boosting proliferation however numerous others points towards adverse effects on redifferentiation (P Giannoni. et al., 2005, L Enochson et al., 2012, R Narcisi et al., 2012). The evidence base will undoubtedly benefit from further contribution.</td>
<td>Yes</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Demonstrated to stimulate growth in chondrocyte gel culture(A H Huang et al. 2009). Little reports of effect on 2D culture to date. As growth factors place high demand on resource, it may be more prudent to wait until more basic research establishes a link between its presence and effect on proliferation/phenotype.</td>
<td>No</td>
</tr>
<tr>
<td>IL-1</td>
<td>Implicated in upregulation of MMPs ( E Kolettas et al., 2001).</td>
<td>No</td>
</tr>
<tr>
<td>IL-6</td>
<td>Implicated as a negative regulator for chondrocyte proliferation and matrix production ( A Jikko et al., 1998).</td>
<td>No</td>
</tr>
<tr>
<td>IL-4</td>
<td>Thought to downregulate pro-inflammatory responses but left out due to cost constraints.</td>
<td>No</td>
</tr>
<tr>
<td>IL-8</td>
<td>Thought to upregulate pro-inflammatory responses and MMPs (K Steinbach et al., 2002).</td>
<td>No</td>
</tr>
<tr>
<td>IL-10</td>
<td>Thought to downregulate pro-inflammatory responses ( G Schulze-Tanzil et al., 2009., K Steinbach et al., 2002) but left out due to cost constraints.</td>
<td>No</td>
</tr>
<tr>
<td>TNFα</td>
<td>Regulator of apoptosis in human articular chondrocytes ( B Carames et al., 2008). Not included to focus on factors that regulate SOX gene expression.</td>
<td>No</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Exogenous IGF-1 thought to protect from apoptosis in ex vivo expansion ( T F Higgins and D B Johnson, 2010) but left out due to cost constraints.</td>
<td>No</td>
</tr>
</tbody>
</table>

The evaluation of candidates in Table 1 give rise to following nine candidates for screening: Glucose, Glutamine, ITS, Seeding Density, Fibronectin, FGF-2, PDGF-BB, EGF, and TGF-β1.
2.0 Pilot Study

A concern from the preliminary work in chapter B is the consequence that low statistical powering can have on clearly identifying effects. Judging the size of the expected effect can aid in conducting a prospective power analysis that will allow a more measured estimate of the required sample size. Effect sizes can be judged by using existing literature accounts or by assigning a value that is deemed of interest to the investigator. Unfortunately, neither approach were thought suitable in this case, so a pilot experiment was set up to assess the size of the effect sizes detectable in a range of outputs.

A pilot experiment was conducted to gauge the magnitude of effect sizes that could arise from a run of the main experiment. Defined medium and culture conditions that correspond to the centre point in the proposed fractional factorial experiment were compared to a expansion medium that included FGF-2 and TGFβ-1 in there “+1” states, i.e. are present in higher concentrations. The experiment was batch fed to avoid the possibility of large fluctuations in pH that can occur when re-feeding at seventy-two or twenty-four-hour intervals. The batch approach also served as a cost efficiency. The following outputs were measured: Specific growth rate, glucose consumption, glutamine consumption, lactate production, ammonia production, SOX-9 transcript expression, SOX-6 transcript expression and SOX-5 transcript expression.

2.1 Growth Data

Clonal chondrocytes stored in the vapour phase of liquid nitrogen were reanimated and pre-expanded in defined medium in T175s for five days to reach appropriate cell numbers for the subsequent experiment. Sub-confluent cells were harvested in serum-free conditions and seeded into six-well plates coated in 1µg/cm³ of bovine Fibronectin. Three of
the six wells were supplied with the 0.2mL/cm² of the established defined medium described in Chapter B. This medium is labelled as “Test Condition 2 (-)” in the pilot experiment. The remaining three wells were supplemented with a modified medium that contained 20 ng/mL FGF₂ and 10 ng/mL TGF-β1, labelled as “Test Condition 1 (+). The cells were cultured in batch mode over five days before harvesting. Harvested cells were counted and then sacrificed for RNA extraction as previously described. Medium samples were collected and frozen before metabolite analysis.

![Figure 1. Population doublings achieved by three clonal replicates after five days in culture when expanded under Test Condition 1 or Test Condition 2. Error bars show standard deviation of the mean (n=3).](image)

The three clonal lines achieved under “Test Condition 1”, at the end of the five-day culture, population doublings of 1.41±1.44, 0.37±2.24 and 0.29±0.64 (Fig.1). In contrast to
this, the same three clones cultured under “Test Condition 2” achieved population doublings of 2.28±0.018, 2.06±0.11 and 1.96±0.37 (Fig.1). The population doubling data from this experiment is not directly comparable to the growth data from the batch data in chapter B, as no cell number was recorded for the twenty-four hour time point. Growth data in this experiment was calculated using the seeding density as the starting cell number. In reality, plating efficiency is < 100% as which is why growth data is benefited by early cell number measurements where possible. The data returned from “test condition 2” which, is the previously described defined medium and the proposed centre point in the fractional factorial experiment, shows a narrow distribution. In contrast to the narrowly distributed points from test condition 2, the data set from test condition 1 showed a high level of noise across two of the clonal replicates. The absence of a similar pattern in the same clonal replicates in test condition 2 suggests it is the test condition giving rise to the variation. Variation this large arising from technical replication is of concern. The level of noise has a knock on impact on statistical power, to an end that the degree of replication required to resolve effects may be too great for the resource available. To better understand if the observed variation was a prominent feature of the test condition or special cause case, a further growth study was undertaken.

2.2 Repeated Growth Study

To understand if the variation in growth data seen in 1.1.2 was a repeatable feature of the experimental conditions a further experiment was undertaken. Furthermore, to try and understand any relationship, additional concentration points of TGF-β1 were added to the experiment. The experiment was run with only one clonal replicate. The justification for this lay in the general observation from data in chapter B and from the previous experiment (Fig.
1) that clonal replicates typically behave in the same manner as each other, albeit if the magnitude of the observed behaviour can differ.

Clonal chondrocytes sorted in vapour phase liquid nitrogen were reanimated and expanded in defined medium in T175s for five days to reach appropriate cell numbers for the experiment. Cells were harvested sub-confluent in serum-free conditions and seeded into three 6-well plates coated in 1µg/cm³ of bovine Fibronectin. The three well plates were supplemented with defined medium containing either 20 ng/mL FGF₂/10 ng/mL TGF-β₁ (A), 20 ng/mL FGF₂/5 ng/mL TGF-β₁ (B) or 20 ng/mL FGF₂ (C). The cells were allowed to culture over five days before sacrifice and analysis. No metabolite profiling or gene expression data was recorded from this experiment.

Figure 2. Population doublings from a single clonal replicate treated with defined medium containing three concentrations of TGF-β₁: 10 ng/mL, 5 ng/mL, and 0 ng/mL after five days in culture. Error bars represent standard deviation of the mean (n=6)
The data from the experiment failed to show the degree of variation from the technical replication seen in the original experiment. Interestingly, the average value of the population doublings (Fig.2) of all biological replicates from test condition 1 is approximately proportional to the results seen here (0.73 and 0.13 day\(^{-1}\), respectively). The data also suggests TGF-β1 has a small impact on boosting the rate of proliferation in clonal bovine chondrocytes, which, is perhaps not unexpected given the reported contribution of the protein to chondrocyte proliferation during skeletal growth (F Beier et al., 2001).

2.3 Metabolite Data

Medium samples taken at day five from the pilot study were screened for glucose and glutamine consumption and lactate and ammonia production. Blank measurements were taken from the starting medium in duplicate, and the average used as a reference to point to calculate net consumption and specific consumption/production rates for the five day culture period. Cedex assays from the samples were run as single reactions. The samples run were the original growth experiment (1.1.2) and not the repeated study (1.1.3).
Figure 3. Total consumption / production in mM/L of (A) glucose, (B) lactate, (C) glutamine and (D) ammonia after one hundred and twenty hours in culture. Lactate and ammonia concentrations were adjusted by subtracting background concentrations in the medium. Error bars show standard deviation of the mean (n=3)
The metabolic behaviour follows the growth pattern in that condition 2 gives a tightly distributed band of consumption and production rates. In contrast test condition 1 gives a more erratic response especially in the instance of total glucose and lactate concentrations (Fig.3 (A) and (B)). Total ammonia production, in contrast, was relatively stable between the two test conditions (Fig. D).

**Figure 4.** Specific consumption/production rates reported in pmol.cell⁻¹ day⁻¹ for (A) glucose, (B) lactate, (C) glutamine and (D) ammonia from the after the one hundred and twenty hour culture. Error bars show standard deviation of the mean (n=3)
The cell normalised specific fluxes (Fig.4) highlight clonal replicate two as a potential outlier. The cause of this behaviour is unknown and the question of whether this unexplained behaviour is visible in the gene expression data is of great interest. It should be noted that because daily sampling was not featured in the experiment it’s unclear how the specific flux changed from day to day as the cell number changed.

2.4 Gene Expression Data

To assess if the chondrogenic lineage commitment genes were sensitive to the different test conditions mRNA transcript levels were assessed in the same manner as previously described. Briefly, individual PCR reactions were run in duplicate with random replicates further duplicated to check for reverse transcription efficiency. Copy numbers were derived from standard curves and normalised to the GeNorm score calculated from the endogenous reference genes, B2M, ACTB and RPL13.
**Figure 5.** Normalised copy number from the three clonal replicates for (A) SOX-9, (B) SOX-6 and (C) SOX-5. The difference in means from the two test conditions were tested for significance using an unpaired students t test. Each show standard deviation of the mean (n=3) with the exception of clonal replicate 2 in A, B & C where n=2.
Quantitative PCR assays from the pilot experiment answered some key questions. The assays are sensitive to the changes made to the formulation of the medium (test condition 1) and gave rise to a statistically significant detectable change in SOX-9 transcript (Fig.5 (A)) for clonal replicate 1 and 3 and when compared to the same clones expanded in the control medium (test condition 2) (Table 2). SOX-6 returned similar copy numbers between the two test conditions and the differences were not significant (Fig.5 (B)). SOX-5 transcripts showed a small difference (Fig.5 (C)) between the means of the two test conditions, of which one, clonal replicate 3, was found to be significant. The erratic growth kinetics from test condition 1 did not translate into erratic gene expression data which implies the GeNorm control method is providing a robust normalisation of the data. As a fractional factorial screening experiment would be expected to return both high and low yielding cell densities it is crucial that the PCR assays cannot be influenced significantly by the amount of material available.

2.5 Discussion of Pilot Study Data

The presence of TGF-β1 in test condition 1 along with the increased FGF_2 concentration led to a marked and increase in SOX-9 expression. As a known chondrogenic promoter and a common feature of redifferentiation protocols (Malpeli et al., 2004) the result is not surprising. However, it has been reported that the addition of TGF-β1 during the expansion phase harms down stream redifferentiation potential (R.Narcisi, 2014). If the addition of TGFβ1 is indeed detrimental to redifferentiation capacity then this experiment suggests that the cause of this is not from the downregulation of SOX-9.

The three groups of data from the experiment; Growth kinetics, metabolic flux and gene expression demonstrated that the cell growth was the most erratic output variable and the gene expression was the most stable. Powering of the experiment can be based off any of
the output variables but the decision was taken to estimate an appropriate sample size based off the gene expression results. Reasoning for this lay in the investigators view that the factors the promote the maintenance of the gene profile of interest takes priority over the factors that affect growth rate and the related metabolic flux behaviours.

3.0 Priori Power Analysis

The principal aim of the pilot experiment was to put a range on observed effect sizes to inform a priori power analysis which in turn would suggest an appropriate sample size for the desired power. Of the two key outputs, cell number and gene expression, it was decided that the powering would be based off gene expression results due to the endogenous controls being able to minimise the effect of final cell number on the result. The qPCR data (Fig. 5 (A, B and C)) was used to estimate the size of the effects observed from the pilot experiment in table 2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Clonal Replicate</th>
<th>Test Condition 1</th>
<th>Test Condition 2</th>
<th>Cohen's d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (M) St.Dev (SD) Sample Size</td>
<td>Mean (M) St.Dev (SD) Sample Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX-9</td>
<td>1 735 107 3</td>
<td>216 45 3</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 1069 120 2</td>
<td>443 27 3</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 853 275 3</td>
<td>340 87 3</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>SOX-6</td>
<td>1 149 89 3</td>
<td>72 52 3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 256 99 2</td>
<td>274 49 3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 80 43 3</td>
<td>153 74 3</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>SOX-5</td>
<td>1 219 82 3</td>
<td>310 78 3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 275 91 2</td>
<td>399 9 3</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 204 57 3</td>
<td>334 38 3</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>
An effect size above 1 is defined as a very large effect and anything 0.2 or less as negligible or nonexistent (S. Sawilowsky, 2009). The effect sizes obtained in table 2 were used as a guide to estimate the sample size required for the smallest size effect of interest at a minimum of 80% power at the 0.05 alpha level using a power simulator.

Figure 6. A priori power curve generated using G*Power 3.0.10. Plotted is total sample size versus power for three different effect sizes.

The power curve (Fig. 6) suggests that a sample size of one hundred and twenty should allow an effect size as small as 0.4 to be detected with a power of 0.9. A total sample size in this region was deemed to be just deliverable with the resource available and so this was the perspective sample size taken forward to the DOE stage.
Estimating sample sizes through a pilot study, as done here, has the advantage of delivering robust data on which to base powering decisions when obtaining the same information from the literature is either not possible or problematic. Pilot studies like this also come attached with their own distinct disadvantages. Of chief concern is the fact that the factors altered to create the test condition, TGF-β1, and FGF-2, were selected based on literature accounts that they would induce a change. The magnitude of the change in transcript levels here was, on average, large; however other conditions that are to be included in the screening may produce a real change of a much smaller magnitude. These smaller changes may go undetected, and so it is noted that basing the powering decisions on this pilot study may come at the risk of increasing the incidence of recording a “false negative” or type II error.
4.0 Design of Experiment (DoE) – Fractional Factorial Screening Experiment

4.1 Growth Rate

The growth rate was assessed at the end of the five day culture period. Seeding efficiency was assumed to be 100%. The data was fed into the Design Expert™ 7.0 design matrix without a power transform before been subject to manual regression. Some terms not selected in the manual regression process were automatically included to preserve model hierarchy.
4.1.1 Specific Growth Rate

Figure 7. A pareto chart showing significant factors that influence specific growth rate of clonal chondrocytes over a five day culture. The Bonferoni limit is the threshold for significance. Lack of model fit was deemed not significant (Model F value = 1.39). Curvature (the difference between the centre and factorial points) in the data was deemed significant (F = 15.80).
Figure 8. Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally studentized Residuals and (D) Leverage versus Run.
The Pareto chart (Fig.7) identifies single factors J (Seed Density), H (EGF), F (PDGF-BB), D (Fibronectin), A (Glucose) and G (FGF-2) as significant contributors to the growth rate. Seeding density (J) appearing as the most significant single effect is perhaps intuitive and could be rationalised in terms of, more cells seeded at the start equals a larger proportion of cells successfully attaching and so more cells dividing and contributing to the overall population growth. Higher seeding densities have been previously touted as a method of achieving phenotypic stability in chondrocytes (F M Watt, 1988) however high-density culture can reduce the maximum achievable expansion capacity in traditional flask cultures. By increasing the seed density the maximum number of doublings that can be achieved before the confluency is reached and passaging becomes necessary is lowered. This problem could be mitigated by using expansion platforms that allow for the addition of more growth substrate during the culture period. The three growth factors EGF (H), PDGF-BB (F) and FGF-2 (G) have an important role in stimulating proliferation. To best understand how to employ them in a financially feasible manner, first the shape of the relationship needs to be understood. The curvature detected Fig.7, F=15.80, p=0.0001) means additional exploration of the design space may be required in order to appropriately understand the relationship. Additionally, consideration of the contribution of the growth factors to gene expression is required. Fibronectin coating concentration was a significant effect and again this result might of been predicted on the basis that more binding protein should allow for more cell attachment and subsequently more actively dividing cells. The more interesting discovery is arguably the contribution of glucose (A) to growth rate. Higher growth rates were achieved when glucose (A) was present in its lowest concentration which infers high glucose concentrations are detrimental to the growth of clonal chondrocytes. This finding adds a further view to the findings of other investigators highlighted in table 1.
The results obtained from the model and displayed in the Pareto chart (Fig. 7.) were subject to a number of diagnostic tests (Fig. 8) to test the credibility of the data and the impact of any erroneous data points on the overall output. The normal probability plot of residuals (Fig. 8 (A)) shows a slight “S” like character to the distribution with initial and final data points landing to the left and right of the fitted line, respectively. This implies the distribution has slightly shorter tails then may be expected from a totally normal distribution. It was not expected that the model would fit a normal distribution perfectly and it was decided the deviation from the straight line was within an acceptable limit. The residuals vs run order (Fig. 8 (B)) was interrogated in order to ascertain if there was any time related variable influencing the data. This is especially important given the difference in the time between sacrificing individual experiments and the potential for this to skew the data. The plot displays a random pattern showing that no time-dependent variable was influencing the output data. The externally studentized residuals plot (Fig. 8. (C)) provides an easy visual method of identifying possible outliers in the data set. The plot identified runs 30 and 126 as outliers. The values entered for the runs were checked for errors or any evidence of special cause, but in the absence of either factor, it was concluded that the two data points just did not fit the model that well. Employing a power transform to try and improve model fit did not significantly alter the outcome regarding main effects, and so the data set was left as is. The leverage versus run plot (Fig. 8 (D)) looks at the possibility that an individual design point can significantly influence the fit of the model. The diagnostic failed to highlight any individual data points that were having a disproportionate effect on the model which. The risk of this was lowered due to the incorporation of replicates into the design.
4.2 Metabolic Flux

Glucose, glutamine, lactate and ammonia fluxes were assessed by determining total consumption/production at the end of the culture and relating to the final cell number in the same manner as previously described. The resulting data was added into the design matrix and a model fitted as was done for the growth rate.
4.2.1 Specific Glucose Consumption

Figure 9. A pareto chart showing significant factors that influence specific glucose consumption in chondrocytes over a five day culture. The Bonferoni limit is the threshold for significance. A log power transform was employed. Model fit was deemed significant (Model F value = 19.84, p=<0.0001). Curvature (the difference between the centre and factorial points) in the data was deemed not significant (F = 0.12).
**Figure 10.** Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally studentized Residuals and (D) Leverage versus Run.
The Pareto chart (Fig.10) for specific glucose consumption identifies single factors B (Glutamine), A (Glucose) and H (EGF) as significant contributors to specific glucose consumption. Glucose consumption was highest when glutamine (B) was present in its higher concentration which suggests restricting glutamine does not promote increased glucose usage. Glucose (A) was disregarded as it is both a dependent and independent variable in the study. EGF (H) increases specific glucose consumption, and this is sensible given the previously reported mitogenic activity (Fig. 7)

The diagnostic graphs highlight some concerns with the returned data. The normal probability plot (A) indicates the residuals deviate from a normal distribution and that caution may be needed when interpreting the results. Several power transforms of the data were attempted in order to improve the distribution, but none were successful in doing so. The internally studendised residuals vs run order (Fig. 9 (B)) did not appear to show anytime time-related correlation, however, the externally studendised plot (Fig.9 (C)) highlighted a number of outliers, runs 57,58 and 69. The data for those runs was checked for error, but in the absence of any obvious cause of the unexpected result, it was decided to retain the values in the data set.
4.2.2 Specific Lactate Production

Figure 11. A pareto chart showing significant factors that influence specific lactate production in chondrocytes over a five day culture. The Bonferoni limit is the threshold for significance. Model fit was deemed significant (Model F value = 12.09, p=<0.0001). Curvature (the difference between the centre and factorial points) in the data was deemed to be significant (F = 8.15).
Figure 12. Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally studentised Residuals and (D) Leverage versus Run.
The Pareto chart (Fig.11) for specific lactate production identifies single factors J (Seed Density), H (EGF), A (Glucose) as significant effects. Factor F (PDGF-BB) just fell short of the significance threshold. Specific lactate production was at its highest when seed density (J) and EGF (H) were in their lowest experimental level. In the instance of EGF (H), rationalisation of the result is relatively straightforward. As EGF has mitogenic properties it would follow that lower concentrations may stimulate growth to a lesser extent and decrease the rate of glycolysis resulting in lower lactate production. Explaining why the seed density (J) lower experimental level yielded the higher specific production rate is harder. One explanation is that low seed densities could trigger changes in metabolic behaviour, possibly through paracrine signalling that results in inefficient glucose usage. This high lactate production was encountered in the previous chapter when rates of proliferation stalled. To date this claim is not supported by existing literature accounts.

A power transform of the data was undertaken in order to obtain the data presented in figures 11 and 12, this was done due to the large range of maximum and minimum responses. The normal probability plot (Fig. 11 (A)) indicates the residuals largely conform to a normal distribution although deviation around the tails can be observed. The internally studendised residuals vs. run order (Fig. 11 (B)) did not appear to show anytime time-related correlation. The externally studendised plot (Fig.11 (C)) highlighted some clear outliers, runs 30 and 126 and a possible outlier, run 125. As was done previously, the input value for the runs was inspected for input error. Removing the data points from the set did not drastically alter the conclusion and so they were left in. No individual data points were thought to be casting significant leverage on the model (Fig.11 (D)).
4.2.3 Specific Glutamine Consumption

**Figure 13.** A pareto chart showing significant factors that influence specific glutamine consumption in chondrocytes over a five day culture. The Bonferoni limit is the threshold for significance. Model fit was deemed significant (Model F value = 71.34, p=<0.0001). Curvature (the difference between the centre and factorial points) in the data was deemed to be significant (F = 43.31).
Figure 14. Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally studentized Residuals and (D) Leverage versus Run.
The Pareto chart for specific glutamine consumption (Fig.13) identified significant single factors effects as B (Glutamine), J (Seed Density), F (PDGF-BB), H (EGF) and D (Fibronectin/Adhesion Protein). Glutamine (B) was expected to feature as a main effect given that it is, like glucose, also an independent variable in the experiment. Lower seed densities (J) favoured higher glutamine consumption in a manner not dissimilar to specific lactate production (Fig. 11). Lower fibronectin concentration (D) gave higher glutamine concentrations which is most likely linked to the seed density (J). The lower experimental levels of the mitogenic growth factors PDGF-BB (F) and EGF (H) gave the highest returned glutamine consumption values. A cautionary note, the true specific consumption rate of glutamine is probably obscured by the natural decomposition of the component in the culture environment (O. Palsson et al., 1990).

A power transform of the data was undertaken in order to obtain the data presented in figures 13 and 14. Again this was done due to the large range of maximum and minimum responses. The normal probability plot (Fig. 14 (A)) indicates the normality is a reasonable assumption. The internally studendised residuals vs run order (Fig. 13 (B)) did not show a time-related correlation. The externally studendised plot (Fig.11 (C)) highlighted single clear outliers, run 126 a possible outlier, run 30. As was done previously, the input value for the runs was inspected for input error. Removing the data points from the set did not drastically alter the conclusion and so they were left in. Interestingly though, this is the third time run 126 has flagged as an outlier. No individual data points were thought to be casting significant leverage on the model (Fig.11 (D)).
4.2.4 Specific Ammonia Production

Figure 15. A pareto chart showing significant factors that influence specific ammonia production in chondrocytes over a five day culture. The Bonferoni limit is the threshold for significance.
Model fit was deemed significant (Model F value = 57.80, p=<0.0001). Curvature (the difference between the centre and factorial points) in the data was deemed to be significant (F =6.70).
Figure 16. Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally Studentized Residuals and (D) Leverage versus Run.
The Pareto chart for specific ammonia production (Fig.15) identified almost the same significant single factor effects as for specific glutamine consumption, namely B (Glutamine), J (Seed Density), F (PDGF-BB), H (EGF). Glutamine (B) was expected and the higher experimental level returned the greatest production rates. This will likely be the result of both genuine metabolic production and spontaneous decay of glutamine in the medium (O. Palsson et al., 1990). Again, lower seed densities (J) favoured higher ammonia production. The lower experimental levels of growth factors PDGF-BB (F) and EGF (H) gave the highest returned ammonia production values. Specific production of ammonia, again, is likely obscured by the natural decomposition of glutamine in the culture environment (O. Palsson et al., 1990).

A power transform of the data was undertaken in order to obtain the data presented in figures 15 and 16. The normal probability plot (Fig. 16 (A)) indicates the model distribution is not considered entirely normal. The internally studentised residuals vs run order (Fig. 16 (B)) did not show time-related correlation. The externally studentised plot (Fig.16 (C)) highlighted a sole outlier, run 85, which was left in the data set. No individual data points were thought to be casting significant leverage on the model (Fig.16 (D)).
4.3 Gene Expression Data

SOX-9, 6 and 5 were assessed as described previously. Individual PCR reactions were run in duplicate with random replicates further duplicated to check for reverse transcription efficiency. Copy numbers were derived from standard curves and normalised to the GeNorm score calculated from the endogenous reference genes, B2M, ACTB and RPL13.
4.3.1 SOX-9 Gene Expression

Figure 17. A pareto chart showing significant factors that influence SOX-9 gene expression in chondrocytes over a five day culture. The Bonferroni limit is the threshold for significance. Model fit was deemed significant (Model F value = 55.8, p<=0.0001). Curvature (the difference between the centre and factorial points) in the data was deemed to be significant (F =0.04). A square root power transform (k=1) was employed to reduce the output range.
Figure 18. Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally studentized Residuals and (D) Leverage versus Run.
The Pareto chart for SOX-9 transcript expression (Fig.17) identified that eight of the nine independent variables investigated showed a significant single factor effect. Only factor C (ITS) fell short of the error corrected significance threshold. Factors G (FGF$_2$), E (TGF-$\beta$1) and A (glucose) elicited the highest level on transcript expression when present at their higher levels experimental whereas factors J (seed density), B (glutamine), D (Fibronectin), F (PDGF-BB) and H (EGF) allowed for the greatest transcript expression when present in their lowest experimental concentrations.

The normal probability plot (Fig. 18 (A)) indicates the model distribution again deviates slightly from the straight line in a manner that suggests the data has a short tail but again on balance it was decided that assumption of normality was not totally unreasonable. The internally studendised residuals vs run order (Fig. 18 (B)) did not show time-related correlation. The externally studendised plot (Fig.18 (C)) highlighted no outliers and no individual data points were observed to be casting significant leverage on the model (Fig.18 (D)).
4.3.2 SOX-6 Gene Expression

**Figure 19.** A pareto chart showing significant factors that influence SOX-6 gene expression in chondrocytes over a five day culture. The Bonferoni limit is the threshold for significance. Model fit was deemed significant (Model F value = 15.45, p=<0.0001). Curvature (the difference between the centre and factorial points) in the data was deemed to be significant (F =3.67).
**Figure 20.** Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally studentized Residuals and (D) Leverage versus Run.
The Pareto chart for SOX-6 transcript expression (Fig.19) identified a sole factor, H (EGF) as a significant main effect, although factor B (glutamine) was close to the significance threshold. EGF (H) promoted the highest level of expression when present in its higher experimental dosage.

The normal probability plot (Fig. 20 (A)) showed a small deviation from the regression line. The internally studentised residuals vs. run order (Fig. 20 (B)) did not show a time-related correlation. The externally studentised plot (Fig.20 (C)) highlighted no outliers and no individual data points were observed to be casting significant leverage on the model (Fig.20 (D)).
4.3.3 SOX-5 Gene Expression

**Figure 21.** A pareto chart showing significant factors that influence SOX-5 gene expression in chondrocytes over a five day culture. The Bonferroni limit is the threshold for significance. Model fit was deemed significant (Model F value = 55.8, p=<0.0001). Curvature (the difference between the centre and factorial points) in the data was deemed to be significant (F =0.04).
Figure 22. Diagnostics from the specific growth data analysis. (A) Normal Plot of Residuals, (B) Residuals versus Run Order, (C) Externally studentized Residuals and (D) Leverage versus Run.
The Pareto chart for SOX-5 transcript expression (Fig.21) identified factors, A (glucose), C (ITS) and G (FGF2) as significant main effects. All three of the factors promoted the highest level of expression when present in their higher experimental dosage.

The normal probability plot (Fig. 22 (A)) suggests the data have short tails which is a feature in common between the dependent variable data. The internally studentised residuals vs run order (Fig. 22 (B)) did not show time-related correlation. The externally studentised plot (Fig.22 (C)) highlighted a sole outlier, run 34, which was left in. No individual data points were observed to be casting significant leverage on the model (Fig.22 (D)).
5.0 One Factor Effects Discussion

The fractional factorial design revealed the key single effect contributors to the growth rate of the clonal chondrocytes (table 4). The main positive effects were broadly anticipated, especially in the case of the growth factors that have previously been reported as mitogenic. Arguably the most interesting discovery was the result that shows high glucose (factor A) concentration is detrimental to the growth rate of clonal chondrocytes which adds favour to the low glucose argument made by other investigators (table 1). The choice, however, may not be straight forward when taking it account that high glucose appears to promote SOX-9 transcript expression. The mechanism that underpins this discovery is currently unknown.

The specific metabolic fluxes for glucose, lactate, glutamine and ammonia returned significant single factor effects that could have been reasonably estimated using prior knowledge of the system. No surprising effect was or unexpected effect was uncovered.

The data obtained from mRNA expression levels of the three genes of interest suggest there are a number of variables that contribute to the upregulation of transcript levels. SOX-9 transcript levels were positively influenced by FGF-2, TGF-β1 and glucose concentration. The contribution from TGF-β1 to SOX-9 gene expression is consistent with earlier findings in the pilot study and the literature (G. Coricor, 2016). FGF-2 contribution to SOX-9 mRNA expression has also been previously reported (S. Murakami, 2000) and so the finding was not unexpected although the magnitude of the effect was surprising, greatly surpassing the effect of TGFβ1 as a SOX-9 promoter. Unexpected results arrived in the form of negative impacts detected. The data shows that high seeding densities and fibronectin concentrations decrease SOX-9 mRNA expression. The effect of fibronectin concentration on SOX-9 expression has
been previously reported in the literature (Hardingham et al., 2008). The study agreed with the findings presented herein that lower concentrations improved SOX-9 transcript expression. The reason for this was attributed to lower concentrations limiting cell spreading which subsequently reduces the formation of actin stress filaments. This type of cytoskeleton modification leads to the downregulation of SOX-9. The authors of the study logically suggested higher cell densities combined with low fibronectin coating may further limit cell spreading and loss of SOX-9 expression. The results here, however, do not support high-density cultures with respect to SOX-9 expression conservation. Furthermore, as highlighted earlier, high cell density limits the maximum achievable expansion factor in conventional culture systems (i.e., before maximum confluency is achieved), and so low seeding densities may have a double-edged benefit. PDGF-BB also presented as a factor that decreases SOX-9 expression but which also boosts proliferation. Given that FGF-2 also bolsters that rate of proliferation and does not decrease any of the measured gene transcript outputs it may be worth excluding the growth factor from medium formulations purely as a cost saving. Only a sole factor had an impact on SOX-6 mRNA expression. EGF, already identified as positive factor in stimulating growth and also upregulates SOX-6 expression. No single factor was identified that had a negative impact on expression levels. SOX-5 transcript levels were predominantly affected by glucose and ITS concentrations. The findings resonate with SOX-9 mRNA which also shows a positive relationship with glucose. Somewhat paradoxically though, the growth rate is adversely affected by high levels of glucose. No single factor displaying a negative impact was identified.
Table 4. Summary of all primary effects that surpass the Bonferroni correction threshold. Effects are divided into positive and negative contributions and are listed in order of the magnitude of their contribution. “Positive” effects are defined as those that gave the highest the output response when at the highest of the two experimental levels and “Negative” effects those that returned the highest response when at their lowest experimental level.

<table>
<thead>
<tr>
<th>Output</th>
<th>“Positive” 1 Factor Contributions</th>
<th>“Negative” 1 Factor Contributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Rate</td>
<td>Seed Density (J), EGF (H), PDGF-BB (F), Fibronectin (D), FGF-2 (G)</td>
<td>Glucose (A)</td>
</tr>
<tr>
<td>Specific Glucose Consumption</td>
<td>Glutamine (B), EGF (H)</td>
<td>Glucose (A)</td>
</tr>
<tr>
<td>Specific Lactate Production</td>
<td>Glucose (A)</td>
<td>Seed Density (J), EGF (H)</td>
</tr>
<tr>
<td>Specific Glutamine Consumption</td>
<td>Glutamine (B)</td>
<td>Seed Density (J), PDGF-BB (F), EGF (H)</td>
</tr>
<tr>
<td>Specific Ammonia Production</td>
<td>Glutamine (B)</td>
<td>Seed Density (J), PDGF-BB (F), EGF (H)</td>
</tr>
<tr>
<td>SOX-9 gene expression</td>
<td>FGF-2 (G), TGF-β1 (E), Glucose (A)</td>
<td>Seeding Density (J), Glutamine (B), Fibronectin (D), PDGF-BB (F), EGF (H)</td>
</tr>
<tr>
<td>SOX-6 gene expression</td>
<td></td>
<td>EGF (H)</td>
</tr>
<tr>
<td>SOX-5 gene expression</td>
<td>Glucose (A), ITS (C), FGF-2 (G)</td>
<td></td>
</tr>
</tbody>
</table>
6.0 Two Factor Interaction Effect Discussion

The most distinguishable feature of the significant interactions effects is the role of glucose (Table 5). Glucose interacts with other factors in such a varied manner that it makes the data difficult to interpret. It is difficult to say whether higher or lower levels of glucose would be of most benefit, so opting to keep levels at their centre point values (1g/L) would appear the most logical choice. The interaction effect between glucose and glutamine is apparent in the instance of SOX-9, and SOX-6 mRNA expression were overloading the culture medium with the two growth substrates contributes to downregulation of expression. Finally, the Glucose-PDGF-BB (A-F) interaction while bolstering growth rate had an adverse effect on SOX-9 expression. Since more potent promoters of proliferation like EGF have been identified further weight is added for the case to remove PDGF-BB from optimised formulations. It should be noted that because some runs of the experiment were lost not some two-way interactions were confounded and so potential relationships may have been missed.
Table 5. Summary of all two-factor interactions that surpass the Bonferroni correction threshold.

Interactions are divided into positive and negative contributions and are listed in order of the magnitude of their contribution. “Positive” effects are defined as those that impacted the output the most when at the highest experimental level and “Negative “effects those that had the most impact when at their lowest experimental level.

<table>
<thead>
<tr>
<th>Output</th>
<th>Positive 2 Factor Interaction Contributions</th>
<th>Negative 2 Factor Interaction Contributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Rate</td>
<td>Glucose - PDGF-BB (A-F)</td>
<td>Glucose – Seed Density (A-J)</td>
</tr>
<tr>
<td></td>
<td>Glutamine - Fibronectin (B-D)</td>
<td>Glutamine – TGFβ1 (B-E)</td>
</tr>
<tr>
<td></td>
<td>Glutamine – FGF2 (B-G)</td>
<td>Glucose – FGF2 (A-G)</td>
</tr>
<tr>
<td></td>
<td>Glutamine – Fibronectin (B-D)</td>
<td></td>
</tr>
<tr>
<td>Specific Glucose Consumption</td>
<td>Glucose-Seed Density (A-J)</td>
<td>Glutamine-Seed Density (B-J)</td>
</tr>
<tr>
<td>Specific Lactate Production</td>
<td>Glucose-Seed Density (A-J)</td>
<td>Glucose - PDGF-BB (A-F)</td>
</tr>
<tr>
<td></td>
<td>Glutamine - Fibronectin (B-D)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose – FGF2 (A-G)</td>
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<td></td>
<td>Glucose – Fibronectin (A-D)</td>
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<tr>
<td></td>
<td>Glucose – Fibronectin (A-D)</td>
<td>Glucose - PDGF-BB (A-F)</td>
</tr>
<tr>
<td></td>
<td>Glutamine – TGFβ1 (B-E)</td>
<td>ITS-Fibronectin (C-D)</td>
</tr>
<tr>
<td></td>
<td>Glucose – Fibronectin (A-D)</td>
<td>Glutamine - PDGF-BB (B-F)</td>
</tr>
<tr>
<td></td>
<td>Glucose – FGF2 (A-G)</td>
<td>Glutamine-Seed Density (B-J)</td>
</tr>
</tbody>
</table>
### Chapter D Summary

The screening of the nine candidates for effect on growth rate and SOX9,6 & 5 mRNA identified EGF and FGF2 as the most prolific promoters of growth and SOX-9,6 & 5 transcript expression. The data for the effect of the nine independent variables on metabolic behaviour uncovered no significant relationships of interest.

The current concern with the output data is that the model reported significant curvature in the majority of the outputs. Navigation of the design space using a linear model is not justified and so further experiments that examine the shape of the relationship are warranted. Further to this, the timing of the addition of mitogenic components into the medium is of interest.
CHAPTER E: TIME-DOSE STUDIES OF THE EFFECT OF FGF-2 & EGF ON GROWTH KINETICS.

1.0 Introduction

The fractional factorial experiment undertaken in Chapter C highlighted a number of medium components that have a direct impact on both growth kinetics and SOX gene expression. A drawback of factorial experiments, however, is that the predictive capability of the resulting model is bound by the limits of the design space. In this instance of the screening experiment, growth factor limits were set as zero for the lower limit and 10ng/mL for the upper limit. To understand the impact of increasing the dose concentration or the frequency of the dose, further experiments need to be undertaken. Rather than study both the dose-response and the time-dose response together it was envisaged that results would be easier to interpret by studying the two separately. The aim here was to gain understanding that will allow the minimum amount of growth factor to be introduced into the medium at time points that will allow for maximal boost in growth rates whilst maintaining SOX expression.
2.0 EGF and FGF-2 Dose-Response Studies on Growth Kinetics

The effect of varying the dose on growth rate was assessed by means of a seven-point concentration series. The growth factor under study was added to the chemically defined medium as a bolus dose. A single vial of cryopreserved clonal chondrocytes was thawed and expanded in a T175 over five days prior to seeding in six-well plates. The pre-expansion was not necessary to achieve workable cell numbers but was undertaken for continuity with previous experiments. Prior to seeding, six-well plates were pre-coated with 0.5µg/cm\(^3\) of bovine fibronectin and placed in an humidified incubator for two hours. Seven batches of chemically defined medium were prepared as previously described with the exception of the growth factor under investigation. EGF or FGF-2 was then added to give concentrations of 100ng/mL, 80ng/mL, 60ng/mL, 40ng/mL, 20ng/mL, 10ng/mL (control) and 1ng/mL. Clonal chondrocytes were seeded at density of 3000 cells/cm\(^2\) and supplied with 0.2 mL/cm\(^2\) of the relevant medium. Cells were cultured over five days in a humidified, 5% CO\(_2\) atmosphere incubator maintained at 37\(^\circ\)C in a fed-batch scenario with respect to the medium components not under investigation. Sacrificial cell counts were taken at twenty-four and seventy-two hours to provide the growth data.
2.1 EGF Dose-Response Study

Fig. 1 Cumulative population doublings in response to varying the medium concentration of EGF. 1 ng/mL (A), 20 ng/mL (B), 40 ng/mL (C), 60 ng/mL (D), 80 ng/mL (E) and 100 ng/mL (F) were compared to the standard dose of 10 ng/mL. Sacrificial cell counts were taken at twenty-four, seventy-two and one hundred and twenty hours. Error bars show standard deviation of the mean (n=3)
EGF was found to be the most mitogenic growth factor in the previous chapter. However, the mitogenic capacity of EGF in the clonal chondrocyte strains does not display a dose-response behaviour with a 1ng/mL dose found to be no more effective at stimulating proliferation as a 100ng/mL dose (Fig. 1). There are, however, confounding factors that cloud the result. The mitogenic action of EGF has previously been reported to be dependent on modulation of EGF receptors by dexamethasone in human diploid foreskin cells (J B Baker et al. 1978) and the possibility of the same relationship existing with these clonal chondrocytes cannot be discounted. Dexamethasone is present in the chemically defined medium in low concentrations (1x10⁻⁷ M) and so by increasing the level, it may be possible to observe a dose-response behaviour in the ranges experimented with here. Another consideration is that the data displayed could be considered relatively noisy and so the power to detect true change

**Fig.2** Specific growth rates for time 24 h – 120 h (A) and 24 h – 72 h (B) obtained for the various EGF concentrations. No statistically significant difference between the control of 10ng/mL and any concentration of EGF used for either of the growth rates. Errors bars show standard deviation of the mean (n=3)
is low. The final consideration is the ill-defined half-life of the EGF protein under culture conditions. The twenty-four feed experiments in Chapter B demonstrated increased exposure to fresh medium promoted proliferation and one explanation to this is that the half-life of EGF in culture is less than twenty-four hours. In this case, increasing the concentration would have little effect on a five-day fed-batch culture. This could be commercially significant as adding frequent low doses of growth factor may be economically advantageous over adding a large bolus dose.
2.2 FGF-2 Dose-Response Study

Fig. 3: Cumulative population doublings in response to varying the medium concentration of FGF-2. 1 ng/mL (A), 20 ng/mL (B), 40 ng/mL (C), 60 ng/mL (D), 80 ng/mL (E) and 100 ng/mL (F) were compared to the standard dose of 10 ng/mL. Sacrificial cell counts were taken at twenty-four, seventy-two and one hundred and twenty hours. Error bars show standard deviation of the mean (n=3)
FGF-2 has previously been previously cited as a growth-promoting agent in auricular chondrocytes (J D Richmon et al., 2005), a redifferentiation promoting agent when used in high doses (E W Mandl et al., 2002) and as a direct intervention agent in the repair of hyaline cartilage defects (P Cuevas et al., 1989). The data collected in this experiment failed to identify a statistically significant dose-response relationship however, visually it would appear there is weak evidence for the existence of a dose-response that may warrant further investigation. Compared to the 10 ng/mL control there is a small increase in population doublings and growth rate with each subsequent increase in FGF-2 concentration. A one way ANOVA was performed which showed a difference between the means existed. Post hoc T-tests were used to try and establish where the significance existed. P values were less than 0.05 for 80 ng/mL and 100 ng/mL but did not meet significance criteria after a conservative
family-wise error correction was applied. One observation of interest is that the 1 ng/mL dose performed at least as well as the 60 ng/mL dose and a similar result was observed in the EGF dose series. The reason for this is not immediately clear. Possible explanation may be an experimental error incurred during dose preparation; the 1 ng/mL dose preparation entailed pipetting a small volume of 0.2 µl into a large 30 mL volume. It is possible improper mixing may have effectively resulted in no growth factor been presented to the cells. Such a scenario lays way to a more perplexing conundrum however, why would no FGF-2/EGF yield a better proliferative response than the standard 10 ng/mL supplemented medium. Further experimental investigation will help answer this question by the inclusion of a 0 ng/mL control. The experiment is further hampered by low statistical power, the result of financial constraints, which leaves us in the situation where a mitogenic FGF-2 dose response is suspected but cannot be robustly backed up the data.

Establishing a dose-response relationship represents one important step towards an informed feeding regime but equally as important is to try and time resolve the addition of the key medium components. As demonstrated in chapter B, the time at which a feed takes place has a profound impact on the growth kinetics and being able to replace only components of the medium that are required for optimal growth at a given time would be advantageous.
3.0 EGF and FGF-2 Time- Dose Impact on Growth Kinetics and SOX Expression Study

To assess how the timing of the addition of EGF and FGF-2 to the culture medium impacted growth kinetics and SOX-9,6 & 5 gene expression a time-dose study was conducted. A vial of cryopreserved clonal chondrocytes was thawed and pre-expanded under defined conditions for five days to achieve sufficient cell numbers. Harvested cells were seeded into six-well plates that had been precoated with bovine fibronectin (1µg/cm²). Plates were seeded and labelled according to the frequency of feeding. Wells were supplemented with specific medium for the growth factor under investigation. Medium for the EGF study lacked EGF, which was introduced separately at the required time. The EGF medium still contained all other components of the chemically defined medium including FGF-2. The FGF-2 medium contained the base level of EGF with the FGF-2 introduced as stipulated by the experimental condition. Twenty-four-hour dose feeding commenced with the addition of the specified growth factor at time 24h and not time zero, this was the case for the forty-eight-hour dose feed as well. The rationale behind delaying the growth factor addition was to assess the impact of immediately exposing the cell strain to the growth factor, as is the case in the fed-batch scenario. A negative experiment was also set up to investigate the observation from the dose-response study that suggested little to or no EGF/FGF-2 could be as beneficial as higher dosages.
3.1 EGF Time-Dose Studies on Growth Kinetics

**Fig. 5** Cumulative population doublings in response to addition of EGF (20 ng/mL) at twenty-four hour intervals (A) and forty-eight hour intervals (B). An EGF negative experiment was run (C). Graph D displays all data points on the same axis for comparison. Sacrificial cell counts were taken at twenty-four, seventy-two and one hundred and twenty hours in order to obtain the growth curve. Error bars show standard deviation of the mean (n=3)
The results from the EGF dose frequency experiment were unexpected and perplexing. On the one hand, the data demonstrated that the addition of EGF at twenty-four-hour intervals yielded the greatest growth rate of all the test conditions and a statistically significant difference over the fed-batch approach. On the other hand, the absence of EGF from the culture medium resulted in a similar growth rate to that of the twenty-four group. The result was also found to be statistically significant when compared to the fed-batch group. Keeping the two facts separate for now makes interpreting the results simpler. Cell growth appears to respond positively but weakly to the addition of EGF into the culture medium. Interrogation of the fed-batch growth curve gives way to a common feature of growth curves obtained from fed-batch experiments, that is, the growth curve tends to plateau around the seventy-two-hour mark, which can be averted by addition of EGF at twenty-four

![Fig.6](image)

**Fig.6** Specific growth rates for the EGF dose frequency experiment over one hundred and twenty hours (A) and over seventy-two hours (B). A one-way ANOVA was performed and indicated there was a significant difference between the means. Post hoc t tests showed significance between the 24h dose and EGF negative compared to Fed batch after the Bonferroni correction was applied.
or forty-eight-hour intervals. This observation occurs without the culture reaching
confluency. A possible explanation for this, as stated earlier, is the half-life of EGF under
culture condition is in the region of seventy-two hours. Further, the data suggests there is not
only any advantage to be had in terms of stimulating proliferation from exposing the clonal
strains to EGF at the time of seeding but doing so actually harms the initial growth phase.
The proliferation in the “EGF negative” experiment is driven by the FGF-2 and the PDGF-
BB, both weaker mitogens than EGF as inferred by the DOE. The growth curve again shows
a plateau in growth in a manner that is almost identical to that of the fed-batch experiment.
This would again point to the stability of the FGF-2 and PDGF-BB proteins in the culture
medium beyond seventy-two hours. Explaining why the EGF negative experiment resulted in
the greatest number of population doubling at seventy-two hours requires a greater degree of
speculation. The result could be due to a two or more factor interaction, likely between the
other growth factors that give rise to a “too many cooks” type scenario during the initial
stages of the culture. This appears to be the first time this behaviour has been reported but to
understand the mechanism further studies would be required. It should be noted that the two-
factor interactions between EGF and FGF-2 and PDGF-BB were aliased in factorial
experiment and so if such an interaction did exist, has been missed.

Growth factors are generally expensive, delaying the addition of EGF until a later
stage in the culture may be considered efficient or least that is until you consider the result of
the EGF negative experiment. The impact of dose frequency on SOX expression needs to be first assessed before concluding how EGF is best employed in a defined culture medium.

3.2 EGF Time- Dose Studies on SOX Gene Expression

Clonal chondrocytes from all four experimental conditions were harvested after one hundred and twenty hours. Half of the harvested cells were suspended in RNA Cell Protect™ and stored at -20°C until the RNA extraction step was ready to be performed. RT-qPCR was performed to determine gene transcript levels of SOX-9, 6 & 5. Gene expression levels from the EGF negative, EGF 24h and EGF 48h were compared to the Fed-batch results.

![Fig. 5 SOX-9 mRNA transcript copies from clonal chondrocytes expanded over five days with different EGF interventions imposed. mRNA transcripts were determined using RT-qPCR. The differences between the groups were not found to be significant (p=0.05) using a one-way ANOVA. Error bars show standard deviation of the mean (n=3)]
Fig. 6 SOX-6 mRNA transcript copies from clonal chondrocytes expanded over five days with different EGF interventions imposed. mRNA transcripts were determined using RT-qPCR. The differences between the groups were not found to be significant ($p=0.05$) using a one-way ANOVA. Error bars show standard deviation of the mean ($n=3$).

Fig. 7 SOX-5 mRNA transcript copies from clonal chondrocytes expanded over five days with different EGF interventions imposed. mRNA transcripts were determined using RT-qPCR. The differences between the groups were not found to be significant ($p=0.05$) using a one-way ANOVA. Error bars show standard deviation of the mean ($n=3$).
SOX-9 gene expression was not found to be positively affected by the presence of EGF to a statistically significant extent like in the screening experiment detailed in chapter B. The data in Fig.5 shows the EGF fed-batch group resulting in a higher mean average of SOX-9 transcripts when compared to the EGF negative group. SOX-9 expression was at the highest level in cultures exposed to twenty-four hour EGF dosing. The variability observed creates a high degree of noise, which makes statistical comparison difficult at low replicate number, a feature shared with the kinetic growth experiments too. Like SOX-9, SOX-6 was shown to be upregulated by EGF. The data here again was not found to be statistically significant however; Fig. 6 shows a weak correlation in that the addition of EGF at time zero and more frequent EGF addition leads to higher SOX-6 transcripts. The screening experiment was designed to be more statistically powerful than the experiment here which is likely why the difference between means fall just outside of the significance threshold here. EGF was not found to make any difference to SOX-5 expression in the screening experiment and here the data is absent any form evidence to the contrary.
3.3 FGF-2 Time-Dose Studies on Growth Kinetics

**Fig. 8** Cumulative population doublings in response to addition of FGF-2 (20 ng/mL) at twenty-four hour intervals (A) and forty-eight hour intervals (B). An FGF-2 negative experiment was run (C). Graph D displays all data points on the same axis for comparison. Sacrificial cell counts were taken at twenty-four, seventy-two and one hundred and twenty hours in order to obtain the growth curve. Error bars show standard deviation of the mean (n=3)
The impact of varying the frequency of FGF-2 addition into the culture was assessed in much the same manner as EGF. The differences between mean growth rates of the different experimental groups did not show statistical significance. A weak visual trend provides some points for debate. Firstly, the more frequent addition of FGF-2 the higher the growth rate over a one hundred and twenty hour period. The screening experiment showed that FGF-2 had the weakest mitogenic effect of the growth factors and so, as the power in this experiment is lower, it follows that the relationship that forms here falls short of the significance threshold. The experiment here requires more replicates to increase the power in order to confirm the effect is genuine. The next point of interest is the apparent stability of FGF-2 versus EGF under culture conditions. The FGF-2 negative group contains EGF and it follows that the EGF and PDGF-BB are driving the proliferation up until seventy-two hours where the population starts to decline. The FGF-2 fed-batch control continues to support
proliferation after this time. A similar trend was observed in the EGF-dose frequency experiment, which adds weight to the notion that the EGF protein has a half-life of seventy-two hours under the culture conditions reported here. The most striking feature of the data collected here is the difference in growth observed between the FGF-2 negative group and the FGF-2 fed-batch group. Similar to the EGF experiment it appears withholding the addition of FGF-2 at the start of the culture results in enhanced proliferation in the first seventy-two hours of culture. Like with EGF, the impact of varying the frequency of FGF-2 dosing on SOX-9,6 and 5 gene expression was assessed. The two proteins, EGF and FGF-2, have a negative interaction effect in early stage culture, which is overcome as time increases.
3.4 FGF-2 Time- Dose Studies on SOX Gene Expression

Clonal chondrocytes from all four experimental conditions were harvested after one hundred and twenty hours. Half of the harvested cells were suspended in RNA Cell Protect™ and stored at -20°C until the RNA extraction step was ready to be performed. RT-qPCR was performed to determine gene transcript levels of SOX-9, 6 & 5. Gene expression levels from the FGF-2 negative, FGF-2 24h and FGF-2 48h were compared to the fed-batch results.
**Fig. 5** SOX-9 mRNA transcript copies from clonal chondrocytes expanded over five days with different frequency FGF-2 interventions imposed. mRNA transcripts were determined using RT-qPCR. The differences between the mean of the fed-batch and the three groups were all found to be significant using student’s t test (equal variance) with a Bonferroni error correction (p=0.016). Error bars show standard deviation of the mean (n=3).

**Fig. 5** SOX-6 mRNA transcript copies from clonal chondrocytes expanded over five days with different frequency FGF-2 interventions imposed. mRNA transcripts were determined using RT-qPCR. The differences between the mean of the fed-batch and the three groups were all found to be significant using student’s t test (equal variance) with a Bonferroni error correction (p=0.016). Error bars show standard deviation of the mean (n=3).
The gene expression analysis allowed for some interesting observations. SOX-9 mRNA transcripts were positively impacted by the presence of FGF-2 which is not surprising since the screening experiment and existing literature both demonstrate this (E Mandl et al. 2002). Interestingly, to achieve a significant increase in SOX-9 transcripts the data suggests that FGF-2 has to be present at the time of inoculation, twenty-four hour and forty-eight dosing was not found to be any more effective at upregulating expression than the FGF-2 negative group.

The data from SOX-6 expression shows a clear trend of transcript decline with increased dosing of FGF-2. This negative impact of FGF-2 on SOX-6 mRNA expression was
observed during screening but the relationship was disregarded because it failed to pass the Bonferroni corrected threshold for significance. This highlights a drawback of using this method of familywise error correction in that when large comparisons are made the risk of a type II error or false negative increases. The absolute impact of harming SOX-6 expression with the use of FGF-2 is not known. Whilst upregulation of SOX-9,6 & 5 is thought to be one of the keys to allowing the efficient induction of mature cartilage, the exact impact of SOX-6’s contribution to this paradigm is not fully understood.

The returned data from the SOX-5 gene expression returned the most interesting feature and perhaps a cautionary lesson regarding the interpretation of data from DoE models alone. The data here showed that the presence of FGF-2 in the fed-batch culture significantly increased transcript expression over the negative group, which is shown clearly in the screening data. What might not be expected from the screening data is that continued addition of FGF-2 to the system has the opposite effect and actually reduces SOX-5 transcript levels to below that recorded for the FGF-2 negative group. A limitation of the DoE model is that the relationships are, at best, only valid within the fixed limits of the design space and that extrapolating trends beyond the original parameters is not sensible.
4.0 Summary of Chapter E

The data from the dose and dose frequency experimenting revealed in more detail, the role of EGF and FGF-2 in the expansion of the clonal chondrocytes. The question of how best to employ the proteins in a manner that best promotes growth, maintenance of SOX expression and cost efficiency is not straightforward. The experiments suggest that the introduction of both EGF and FGF-2 at the time of cell seeding is detrimental, resulting in an increased lag phase that can be mitigated in late-stage culture with increased frequency of EGF dosing. FGF-2 did not show the same level of mitogenic activity as EGF here but is a clear driver of SOX gene regulation in these clonal strains. Key points are summarised in Table 1.
**Table 1. Summary of key points from the experiments in this chapter**

<table>
<thead>
<tr>
<th></th>
<th>Dose Response on Growth Kinetics</th>
<th>Dose Frequency Response on Growth Kinetics</th>
<th>Dose Frequency Response on SOX-9, 6 &amp; 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGF</strong></td>
<td>No significant difference between 1 ng/mL – 100 ng/mL range when added as a bolus dose at time of seeding.</td>
<td>24h dosing of EGF significantly increased growth rate over the 120h culture compared to batch fed. Growth rate over 72h was not significant however; the EGF negative group was significantly different when compared to the fed-batch group.</td>
<td>No significant effect detected</td>
</tr>
<tr>
<td><strong>FGF-2</strong></td>
<td>ANOVA identified a significant difference between the means but error corrected post hoc testing failed to pinpoint between which groups the difference existed. Strongest difference was between the 10ng/ml and 100ng/mL. Weak evidence of a dose-response effect.</td>
<td>No beneficial effect of increasing the frequency of FGF-2 dosing on growth rate within the 120h observation window. A larger dose at time of inoculation may harbour an advantage.</td>
<td>Varied effects observed: SOX-9 – most effective when introduced at time of seeding. SOX-6 – Negatively affects expression. The more frequent the dose the greater the negative effect. SOX-5 – Evidence increased dosing negatively affects expression. Fed-batch superior to the EGF negative group.</td>
</tr>
</tbody>
</table>
The points summarised allow a speculative partially optimal nutritional regime to be proposed. A basal medium containing all the components in their nominal concentrations minus EGF and FGF-2 should be formulated. FGF-2 should be introduced as a single bolus dose at the time of cell seeding, evidence for the concentration of the dose is inconclusive but a 100ng/mL may be superior to 10ng/mL with respect to enhancing growth rate. EGF should be withheld from the culture until the seventy-two hour time point where doses of 10ng/mL should be added every twenty-four hours.
CHAPTER F: GENERAL CONCLUSIONS AND FUTURE WORK

1.0 General Summary

The growth of clonal chondrocytes is serum dependent, and the rate of proliferation enhanced by increasing the frequency at which the serum-containing medium is exchanged. Clonal chondrocytes cultured over a five-day period achieved, on average, a specific growth rate of 0.75 day\(^{-1}\) when the serum-containing medium was replaced every twenty-four hours. In contrast to this clonal chondrocytes cultured under the same conditions achieved averaged specific growth rates of 0.63 day\(^{-1}\) and 0.47 day\(^{-1}\) when the frequency of medium exchange was reduced to seventy-two hours and one hundred and twenty hours, respectively. Metabolite analysis of the medium showed that glucose and glutamine depletion were not a rate-limiting factor. Additionally, lactate and ammonia build up were not thought to be significant contributors to the different growth rates obtained. Metabolite analysis did reveal that specific glucose consumption to specific lactate production was higher in the one hundred and twenty hour and seventy-two-hour feed frequency experiments compared to the batch. The results suggest that in the event of the depletion of mitogenic signalling factors present in the serum the cells turn over glucose in an inefficient manner. Serum containing medium was demonstrated to adversely affect expression of SOX-9 transcripts when compared to a defined medium. This is largely thought to be a consequence of the Fibronectin that is present in unknown concentrations in the serum. Interestingly, despite the significant difference in SOX-9 transcripts between the serum and defined cultures, SOX-6 and 5 transcripts were similar suggesting their expression is not tied to same fate as that of SOX-9.
The defined medium formulation, adapted from existing literature accounts, yielded averaged specific growth rates of 0.17, 0.44 and 0.60 day^{-1} for the batch fed, seventy-two hour and twenty-four-hour feed frequency experiments, respectively. The growth rates obtained from the defined medium were inferior to those obtained from using the serum-containing medium. Unlike the serum experiments, there was no obvious pattern in metabolic behaviour with regard to the glucose to lactate conversion ratio. Absolute flux rates in some instances were over an order of magnitude higher than those seen in the equivalent serum experiments. It became apparent the defined medium was sub-optimal for the expansion of clonal chondrocytes although, as already eluded to, SOX-9 transcript expression was better sustained in the defined medium. The defined medium used a tightly controlled amount of fibronectin which was thought, based on the literature, to be the major contributor to this observation. Finally, a cost comparison of the serum containing medium and the defined medium as used in the experiments that the defined culture route cost is approximately nine times greater. The cost is largely attributable to the fibronectin, FGF-2, EGF, and PDGF-BB.

The two-level fractional factorial-designed experiment, employed to understand the effect of nine independent variables on growth rate, metabolic flux, and SOX gene expression, returned both expected and unexpected results. The growth rate was primarily driven by the mitogens EGF, PDGF-BB and FGF-2. Additionally higher seeding densities and fibronectin concentrations contributed to improved growth rates. The higher level of glucose was found to be detrimental to the growth rate and so lower glucose concentrations of approximately 0.5g/L (2.75mM) are recommended if the focus is on optimising growth rate. The metabolic fluxes for glucose, lactate, glutamine and ammonia were determined as a function of the nine candidates. None of the returned data sets provided any major insight into the metabolic behaviour of the clonal chondrocytes; this may be due to the effect size being too small to detect at the power level of the experiment. Further, none of the metabolic
fluxes presented themselves as possible surrogate markers for the conventional SOX quality markers determined by RT-qPCR. Expression of the master chondrogenic promoter gene SOX-9 was affected by nearly all variables in the culture system. The literature suggested that the primary reason behind loss of SOX-9 expression was through the formation of actin stress filament formation (A Woods et al., 2005) which occurs during cell spreading which, in turn, is linked to fibronectin and seed density concentrations. The findings here agree that lower concentrations 0.5µg/cm² of fibronectin promote higher transcript expression however the same is not true of high seed densities, which, have been suggested as a method of reducing cell spreading. The highest SOX-9 expression was obtained with a seed density of 1500 cells/cm². The magnitude of the seed density effect on SOX-9 expression was found to be in the region of approximately double of that observed for the effect of fibronectin concentration on transcript expression. The seed density effect was also found to be roughly proportional to the chondrogenic rescuer TGF-β1 which elicited the highest transcript expression response at its higher level of 10ng/mL. Weighing up the cost-benefit of TGF-β1, a reasonable conclusion would be to remove it from the expansion medium. FGF-2 was determined to be the strongest promoter of SOX-9 transcript expression which is consistent with the literature (S Murakami et al., 2000) although was found to be only weakly mitogenic relative to EGF and PDGF-BB. The use of FGF-2 can serve to counteract the negative effect on SOX-9 expression associated with higher seed densities while benefiting from the boost in growth rate observed in high-density cultures. SOX-6 expression was predominately driven by EGF, which was also identified as one of the more potent mitogens. Additionally, recombinant EGF is relatively cheap and so its use in the culture of clonal chondrocytes is recommended. A significant two-way interaction between glucose and ITS was recorded with the positive effect obtained when both factors were at their higher levels of 1g/L and 20ml/L. It was noted that the single effects were not significant which clouds if this
is a genuine two-factor interaction. With that said, the same interaction was recorded for 
SOX-5 transcript expression, along with significant effects for both glucose and ITS. The 
conclusion then is that the expression of both SOX-6 and 5 can be enhanced using glucose 
and ITS concentrations of 1g/L and 20ml/L, respectively. This also hints that SOX-6 and 5 
transcript expression may be linked to glucose metabolism pathways.

A further problem at this stage is the uncertainty of the shape of many of the 
relationships. As the experiment was only conducted at two levels and significant curvature 
was often detected the effect of altering dosages between the levels is not well understood, 
furthermore, nothing as of yet is known about extending doses beyond the highest level of the 
design. This was addressed by investigating the effect of two factors on growth rate further. 
FGF-2 and EGF were chosen based on the rationale that they, between them, are potent 
promoters of SOX-9 and 6 as well as being significant mitogens. A noted weakness of the 
fractional factorial design was that a number of runs were lost due to assay failure resulting in 
the loss of orthogonality in the design. Consequently, the power to resolve some two-factor 
interactions were lost.

To understand the effect of concentration of both growth factors on growth rate 
beyond the two levels a seven stage concentration series was employed. EGF did not affect 
growth rate in a dose-dependent manner when added as a single bolus dose at the start of the 
culture, with the five-day growth rate obtained from using a 100ng/mL been comparable to 
1ng/mL dose. The growth rate as measured from 24h to 72h showed an overall higher rate of 
growth was achieved earlier in the culture hinting that lack of dose-response maybe owed, at 
least in part, to a short half-life of the EGF protein (Adrian J L Clark, 1985). FGF-2, in 
contrast, did show a dose-response pattern in both 72h and 120h growth rates although. A one 
way ANOVA suggested the difference in obtained growth rates across the concentrations was 
significant although post hoc testing failed to pinpoint exactly where the significance lay. It is
proposed that FGF-2 is more stable under culture conditions which is also supported by the literature (G Chen et al., 2013) and so, as a result, persists longer. Previous data suggested FGF-2 to be a weaker mitogen so again the issue of protein stability and half-life is a plausible explanation. One observation of note was the surprising effect of 1ng/mL compared to higher concentrations, especially on the 72h growth rate. The lower level of the fractional factorial experiment for the growth factors was set at zero. Inefficient or incomplete mixing of the 1ng/mL dose may have led the effective concentration to be zero. It was suspected that maybe a two-factor interaction between the growth factors existed that adversely affected growth rate that could have been missed due to the incomplete design of the previous experiment.

A time-dose experiment was set up to study the effect of introducing the recombinant proteins at specific time points as oppose to bolus dosing on growth rate and SOX-9,6 and 5 gene expression. Twenty-four-hour dosing of EGF (20ng/mL) significantly increased the five-day specific growth rate compared to a single bolus dose. Interestingly, however, so too did the EGF negative control in both five day and seventy-two-hour specific growth rates. The results suggest that EGF does have a profound effect on proliferation but its effect can be hindered by its relatively short half-life in culture (Adrian J L Clark, 1985). Furthermore, there is evidence now of at least a two-factor interaction between EGF and FGF-2 that results in an extended lag phase. The SOX transcript expression results showed no benefit of increased EGF-dosing compared to bolus dosing and again it is proposed this is due to the protein half-life. Timed dosing of FGF-2 revealed that SOX-9 transcript expression was significantly higher when the protein was introduced at the time of seeding, with twenty-four hour and forty-eight-hour dosing not significantly more effective at upregulating transcript expression than the FGF-2 negative control. SOX-6 transcript expression, as discovered earlier, is decreased by FGF-2 addition in a dose-dependent fashion with the lowest average
transcript expression recorded in the twenty-four-hour dose group. SOX-5 transcript expression is best served with using a bolus dose at the time of seeding, however, continued dosing appears to be detrimental to expression with the lowest number of transcripts counted in the twenty-four-hour dose group.

The data returned here reinforces the earlier point that culture parameters that promote proliferation can be counter to the conditions that promote SOX gene expression profiles meaning the ideal culture conditions are a trade-off between the two. It should be noted though that no absolute threshold for SOX transcript expression that translates directly to an efficient expression of the in vivo chondrogenic phenotype was established here. A great deal of further work is required in order to provide a comprehensive culture regime. The role of PDGF-BB dosing as of yet remains unexplored along with the role of pH in the culture system. Outside of the parameters explored here, a great number of other factors and quality markers that were not investigated due to cost constraints remain of high interest. Factors that could replace the use of growth factors are of the highest priority, namely oxygen tension, due to the potential for achieving similar physiological effects without the high cost and possible manufacturing variation.

2.0 General Conclusion

Recommendating a comprehensive medium composition is problematic as it would appear factors that favour proliferation do not necessarily favour upregulation of the chondrogenic promoter genes. A careful balance needs to be considered as to which to give priority to. With the evidence collected in this thesis, a cautionary approach to an optimised and part modulated nutritional regime is presented in table 1.
**Table 1.** Conclusions drawn from the various studies in this thesis on how to maximise growth rate whilst promoting the expression of SOX-9,6 and 5.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.5 mM</td>
<td>A trade-off aimed at enhancing proliferation while promoting SOX-9 transcript expression.</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5mM</td>
<td>Higher glutamine levels were associated with decreased SOX-9 expression and increased ammonia production. The latter may be relieved by switching to a stabilised form of glutamine.</td>
</tr>
<tr>
<td>ITS</td>
<td>20 mL/L</td>
<td>Higher concentrations of ITS supplement did not affect proliferation or metabolic behaviour but was associated with enhanced SOX-5 transcript expression.</td>
</tr>
<tr>
<td>Seed Density</td>
<td>1500 cell/cm²</td>
<td>The lower seed densities returned higher SOX-9 transcript levels but slower growth rates. The slower growth rates maybe acceptable when weighed against the higher expansion factor obtainable when using low seed densities.</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.5µg/cm²</td>
<td>The lower concentration of Fibronectin will significantly reduce the cost of a run and promote SOX-9 transcript expression. This will come at the tradeoff of decreased growth rate.</td>
</tr>
<tr>
<td>FGF-2</td>
<td>20ng/mL added as a single dose at the start of the culture</td>
<td>FGF-2 significantly increased SOX-9 and 5 transcript expression when introduced as a single dose at the start of the culture. Further dosing did not affect growth rate or SOX transcript expression and was detrimental to SOX-6 transcript expression. Larger doses appears to correlate with increased growth rate but the effect on the gene transcript remains unknown.</td>
</tr>
<tr>
<td>EGF</td>
<td>20 ng/mL introduced every twenty-four hours</td>
<td>Timed dosing of EGF did not affect SOX-9,6 and 5 transcript expression over five-day culture but did increase growth rate</td>
</tr>
</tbody>
</table>
after the initial forty-eight hours of culture with the contradiction that this only happened when added when the addition to the culture was separated from FGF-2.

| PDGF-BB | 10ng/mL added as a single dose at the start of the culture | The dose-response range and the effect of time dosing was not investigated. The potential interaction with EGF and FGF-2 remains of high interest. The recommended dose is based on the observed mitogenic effect derived from the study in chapter C. |
3.0 Future Work

A few notable limitations of the studies in this thesis mean the recommendations listed in table 1 need further investigation to increase their robustness. The resolution of the effects of timed dosing of PDGF-BB on the outputs, the possible interaction EGF and FGF-2, as well as its dose-response ceiling all require investigation. Factors not included in the studies offer a source of further optimisations; Oxygen tension and pH are of particular note. The literature provides a strong pretext for the role of oxygen tension in promoting SOX-9 gene expression and would likely prove to be more cost-effective than using recombinant proteins. Studying cell behaviour under true hypoxic conditions is difficult to achieve and requires specialised workstations in order to avoid reintroducing an oxygenated atmosphere when manipulating the cells or administrating diagnostic tests. The work here used bovine chondrocytes due to their accessibility compared to non arthritic human chondrocytes. The consequence of this is that a bridging study between human and bovine cells would be needed if that results here are to be confidently accepted as valid when advancing manufacturing understanding in human chondrocyte lines. A significant but unexplored avenue here is the use of serum in the explant and cryopreservation stages. The bulk of the later work was conducted using serum-free medium but the long-term impact on the cells from the initial exposure to serum remains unknown. To this end, developing a serum-free explant and cryopreservation protocol would be an avenue worth exploring.

The cell culture period was limited to five days in the bulk of the experiments here which means the conclusions drawn here are only valid for that duration. A cell culture platform that uses technology that negates the need for passaging, such as packed bed or microcarrier, would be an ideal platform to further test the robustness of the recommendations here. On top of this, a platform that allows more substrate to be added would open up bioreactor runs well beyond five days. How the growth kinetics and cell
phenotype alter beyond five-day culture is an important question in need of addressing. Further work is needed on assessing the “quality” of the cells. The studies here were limited to only three gene transcripts but in reality, these alone are not enough to sufficiently define the term “quality”. Building a robust quality specification may require additional gene transcript profiling as well as extensive cell proteome profiling, cell subpopulation tracking via cell surface antigen and genetic stability via karyotypic analysis and telomerase expression. Once the theoretical quality specification and cells can be confidently replicated progression to \textit{in vitro} models and eventually patient trials. The greater challenge, however, lies in the development of novel measurement systems that can capture the appropriate metric, either directly or indirectly through surrogate markers, in a low-cost high throughput fashion. The lack of appropriate measurements systems is a major rate-limiting step in the progression of the commercialization of all indication “more-than-minimally manipulated” cell therapies. It’s envisaged that the majority of the limitations discussed here would need to be addressed in order to provide a total manufacturing platform for the cost-efficient and effective expansion of clinically relevant chondrocytes.
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