

## THE INFLUENCE OF NUTRIENT SUPPLY AND CELL DENSITY ON THE GROWTH AND SURVIVAL OF INTERVERTEBRAL DISC CELLS IN 3D CULTURE

Simon Stephan<sup>1,2,3</sup>, W. Eustace Johnson<sup>1,2,4</sup> and Sally Roberts<sup>1,2,\*</sup>

<sup>1</sup>Spinal Studies, Robert Jones & Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, UK

<sup>2</sup>Institute for Science and Technology in Medicine, Keele University, Staffordshire, UK

<sup>3</sup>Research School of Translational Medicine, University of Manchester, Wythenshawe Hospital, Southmoor Road, Manchester, UK

<sup>4</sup>School of Life & Health Sciences, Aston University, Birmingham, UK

### Abstract

The adult human intervertebral disc (IVD) is normally avascular. Changes to the extracellular matrix in degenerative disc disease may promote vascularisation and subsequently alter cell nutrition and disc homeostasis. This study examines the influence of cell density and the presence of glucose and serum on the proliferation and survival of IVD cells in 3D culture.

Bovine nucleus pulposus (NP) cells were seeded at a range of cell densities ( $1.25 \times 10^5$ - $10^6$  cells/mL) and cultured in alginate beads under standard culture conditions (with 3.15 g/L glucose and 10 % serum), or without glucose and/or 20 % serum. Cell proliferation, apoptosis and cell senescence were examined after 8 days in culture.

Under standard culture conditions, NP cell proliferation and cluster formation was inversely related to cell seeding density, whilst the number of apoptotic cells and enucleated “ghost” cells was positively correlated to cell seeding density. Increasing serum levels from 10 % to 20 % was associated with increased cluster size and also an increased prevalence of apoptotic cells within clusters. Omitting glucose produced even larger clusters and also more apoptotic and senescent cells.

These studies demonstrate that NP cell growth and survival are influenced both by cell density and the availability of serum or nutrients, such as glucose. The observation of clustered, senescent, apoptotic or “ghost” cells *in vitro* suggests that environmental factors may influence the formation of these phenotypes that have been previously reported *in vivo*. Hence this study has implications for both our understanding of degenerative disc disease and also cell-based therapy using cells cultured *in vitro*.

**Keywords:** Degenerative disc disease, regenerative therapy, glucose, serum, cell clusters, alginate beads, Ki67, SA- $\beta$ -galactosidase.

\*Address for correspondence:

Sally Roberts  
Robert Jones & Agnes Hunt Orthopaedic Hospital  
Spinal Studies, ARC/TORCH Building  
Oswestry, Shropshire, SY10 7AG, UK  
E-mail: Sally.Roberts@rjah.nhs.uk

### Introduction

The intervertebral discs (IVD) are the main joints of the spinal column and lie between the vertebral bodies. Disorders related to IVD degeneration are common causes of morbidity or deterioration of quality of life (Waddell, 1996). During aging and degeneration, the IVD exhibits extensive morphological changes such as fibrosis of the nucleus pulposus, disorganised lamellae within the annulus fibrosus and calcification of the vertebral endplates (Bernick and Caillet, 1982; Benneker *et al.*, 2005; Roberts *et al.*, 2006b). Some studies have suggested that these changes are the result of tissue remodelling mediated by disc cells (Adams and Roughley, 2006) which occur at a low cell density of approximately 4000 cells/mm<sup>3</sup> in the healthy nucleus pulposus (Maroudas *et al.*, 1975), with increasing death of cells associated with aging and disc degeneration (Gruber and Hanley, 1998; Boos *et al.*, 2002). The metabolism of IVD cells is important to the functioning of the tissue. It has been suggested that nutrient deprivation may reduce cell viability in the disc and therefore be a pathway leading to disc degeneration (Bibby and Urban, 2004; Benneker *et al.*, 2005). Changes in cell behaviour observed in degenerate human IVDs include cell cluster formation (Johnson *et al.*, 2001), cell senescence (Roberts *et al.*, 2006a; Le Maitre *et al.*, 2007) and apoptosis (Sitte *et al.*, 2010). It has been shown that reduced levels of glucose and oxygen, combined with other environmental conditions present in the inner parts of human IVDs (i.e., decreased pH and decreased osmolarity), decreases the anabolic activity of IVD cells (Ohshima and Urban, 1992; Ishihara *et al.*, 1997). The disc's avascularity has long been considered to influence IVD cell function (Maroudas *et al.*, 1975), a view supported by *in vitro* studies.

Few studies, however, have examined the influence of nutrient factors on the proliferation and survival of IVD cells, particularly in a 3D environment. It has been suggested that growth factors may be unlikely to enter the inner regions of the healthy IVD *via* diffusion from the peripheral vasculature (Holm *et al.*, 1981; Grunhagen *et al.*, 2006), since larger, globular proteins are likely to have a lower diffusion rate than smaller, more linear molecules (as has been shown in the cartilage endplate (Roberts *et al.*, 1996)). Disc degeneration is often characterised by increased vascularisation (Kauppila 1995), as well as the appearance of cell clusters that are Ki67 antigen positive, indicating localised cellular proliferation (Johnson *et al.*, 2001). Hence, the provision of serum-derived factors to

IVD cells may increase in pathological IVDs and could play an important role in regulating IVD cell behaviour.

In this study, we have examined the influence of cell seeding density, serum concentration and glucose availability on the growth pattern of bovine IVD cells encapsulated in a 3D sodium alginate culture system to maintain a phenotype more typical of that seen for nucleus pulposus cells *in vivo* than when grown in monolayer (Horner *et al.*, 2002). We show that the growth and survival of bovine NP cells within this system is regulated by these factors in this *in vitro* model, which has given rise to cell phenotypes seen in pathological discs, including cell proliferation and clustering, cell senescence and cell death as seen in pathological IVD.

## Materials and Methods

### Intervertebral disc cells

IVD cells were isolated from the nucleus pulposus (NP) of adult bovine caudal IVD by enzymatic digestion and expanded in monolayer culture in Dulbecco's Modified Eagles Medium (DMEM/F12; Invitrogen, Paisley, UK; catalogue number: 31330-58), containing 3.15 g/L (17.5 mM) glucose and sodium pyruvate (55 mg/L) supplemented with 10 % (v/v) foetal calf serum (FCS), 100 µU/mL penicillin and 100 µg/mL streptomycin, as described previously (Johnson *et al.*, 2008). At passage II, NP cells were encapsulated into sodium alginate (ISP Keltone) beads at final cell densities of  $1.25 \times 10^5$ ,  $2.5 \times 10^5$ ,  $5 \times 10^5$ , and  $1 \times 10^6$  cells/mL with a final alginate concentration of 1.2 % (w/v) and polymerised in 100 mM CaCl<sub>2</sub>. Culture conditions using DMEM F12 medium containing 3.15 g/L glucose and 55 mg/L sodium pyruvate supplemented with 10 % FCS, 100 µU/mL penicillin and 100 µg/mL streptomycin were designated as 'standard' cell culture conditions. NP cells were also cultured with varying concentrations of FCS (0, 10 and 20 %) or in DMEM/F12 without glucose or sodium pyruvate (Catalogue number 11966025). All culture media were at pH 6.9-7.1 and 312-321 mOsm/kg water. Cells were fed at 48 hourly intervals after the initial seeding and maintained at 37 °C with 5 % CO<sub>2</sub> and atmospheric oxygen in all experiments before harvesting on days 0, 4 and 8. (All reagents were from Invitrogen unless otherwise stated). Alginate beads were depolymerised with 10 mM EDTA-PBS and cytopsin preparations (using 1 alginate bead each time) were made by spinning at 500 rpm for 3 min (Shandon Cytospin 2, Thermo Scientific Shandon, Loughborough, UK).

### Cell proliferation, viability and apoptosis

Cell viability within alginate beads was assessed by 'live/dead' scoring (LIVE/DEAD, Invitrogen), where live cells that maintained non-specific esterase activity cleave calcein-AM to produce green fluorescence, but dead cells that have compromised membranes are permissive to penetration of ethidium homodimer, which binds to nuclear DNA and fluoresces red. Cytopsin were stained with haematoxylin and eosin or diamidino-2-phenylindole (DAPI) and cells counted as single cells, paired cells and cell clusters (defined as 3 or more cells; more than 200

cells were counted per sample). Apoptosis was identified morphologically in DAPI-stained cytopsin via pyknosis and karyorrhexis (nuclear fragmentation), scoring the frequency in 200 or more cells. The proliferation status of cells was examined by immunocytochemistry for the proliferation-associated Ki67 antigen. For this, cytopsin were fixed in 10 % formalin for 10 min, washed with phosphate buffered saline (PBS) and subjected to antigen retrieval overnight at 60 °C in 50 mM Tris buffer, pH 8, before incubating with 1:50 v/v monoclonal antibody specific for Ki67 in PBS (Novocastra, Newcastle upon Tyne, UK). Immunoreactivity was detected using a commercial kit (Vectastain ABC Elite, VectorLabs Ltd., Peterborough, UK) combined with 3,3'-diaminobenzidine (DAB) staining (Vector Labs). Isotype-matched irrelevant antibodies (Dako, Ely, UK) were used in place of the primary antibody as a negative control. The proportion of Ki67 immunopositive cells was determined by counting more than 200 cells per sample.

### Cell senescence

Senescent cells were identified by their production of senescence associated-β-galactosidase (SA-β-Gal), which is demonstrated by histochemical staining with the substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside titrated to pH 6.0 (in comparison to lysosomal β galactosidase at pH 4.0) (Dimri *et al.*, 1995). The proportion of SA-β-Gal positive cells was determined by counting at least 200 cells per sample.

### Filamentous actin staining with phalloidin-FITC

Cytopsin were fixed with 10 % formalin for 10 min and washed with PBS, before incubating with phalloidin-FITC (Molecular Probes/Invitrogen, diluted 1:100 (v/v) in PBS) at room temperature for 30 min. Slides were then washed with PBS and mounted in Vectamount containing DAPI to counterstain cell nuclei.

### Cell labelling with cell linker, PKH26

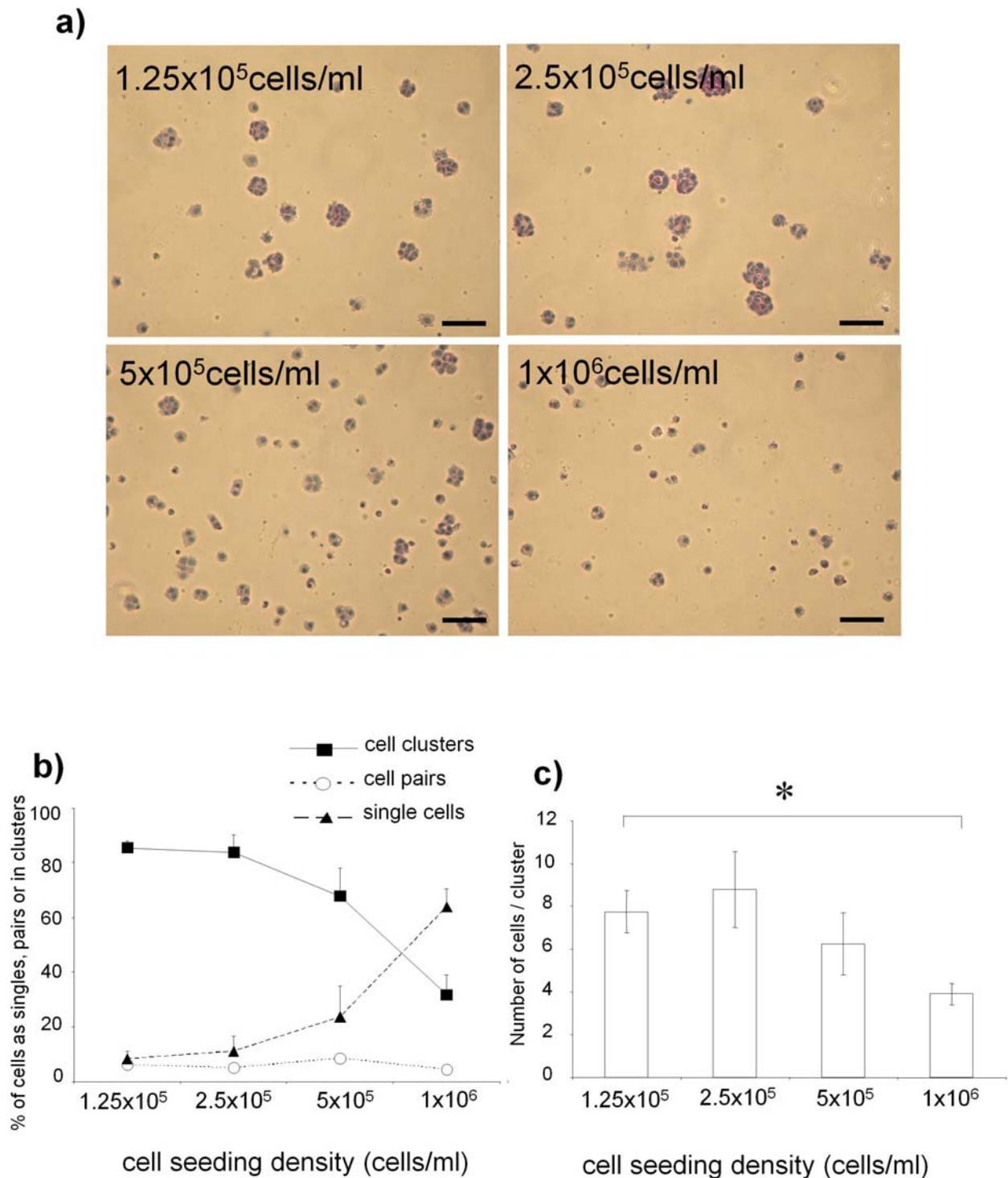
Cytopsin preparations were air dried and washed with distilled water, before staining with the red fluorescent cell linker, PKH26 (Sigma-Aldrich, Gillingham, UK); the dye was diluted 1:200 v/v in buffer, according to the manufacturer's instructions and used at room temperature for 15 min. Slides were then washed with PBS and mounted in Vectamount containing DAPI to counterstain cell nuclei.

### Microscopy and image capture

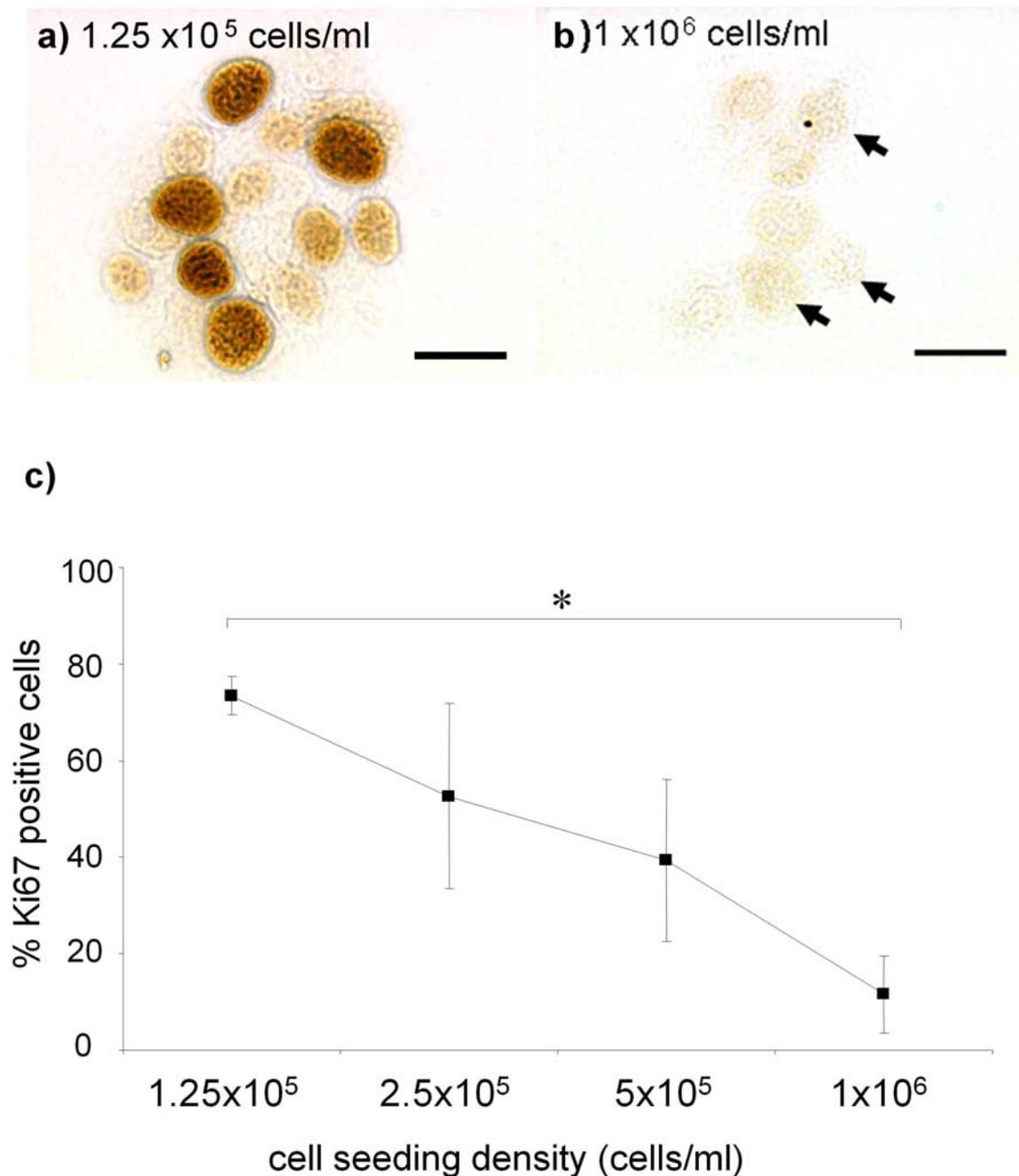
NP cells were viewed, either *in situ* within alginate bead cultures or in the cytopsin using phase contrast and fluorescence microscopy (Nikon Eclipse TS100, Nikon, Kingston-upon-Thames, UK). Digitised images were captured with a Hamamatsu (C4742-95) or Nikon (Coolpix) digital camera and examined using IP Lab software (Version 3.6, Nikon).

### Statistical analysis

Each experiment was carried out in triplicate, with each set of NP cell cultures having been established from tails of 3 different cows (the NP cells being isolated and pooled from 5 discs for each cow). Differences between 2 groups



**Fig. 1.** (a) Representative wide field images showing cell distribution over a range of cell seeding densities ( $1.25 \times 10^5$ - $1 \times 10^6$  cells/mL) after 8 d in culture in alginate beads in standard culture conditions. Cells were stained with haematoxylin and eosin. Scale bars represent  $40 \mu\text{m}$ . (b) Graph shows the mean percentages of cells in clusters, pairs or single cells in alginate beads seeded at different cell densities and cultured in standard conditions ( $\pm$  SD). (c) The average number of cells per cluster also varied with seeding density mean  $\pm$  SD), (\* denotes significantly more cells/cluster ( $p < 0.05$ ) at  $1.25 \times 10^5$  compared to  $1 \times 10^6$  cells/mL).



**Fig 2.** Ki67 immunopositivity of cells grown in alginate beads was greatest in cells seeded at the lower density (a) compared to high cell seeding density (b) (arrows indicate negatively stained nuclei). Scale bars represent 10  $\mu$ m. (c) Percentage of Ki67 positive cells cultured in standard conditions after 8 d  $\pm$  SD of the mean. (\* denotes significantly more cell proliferation ( $p < 0.05$ ) with cells seeded at  $1.25 \times 10^5$  cells/mL compared to  $1.0 \times 10^6$  cells/mL).

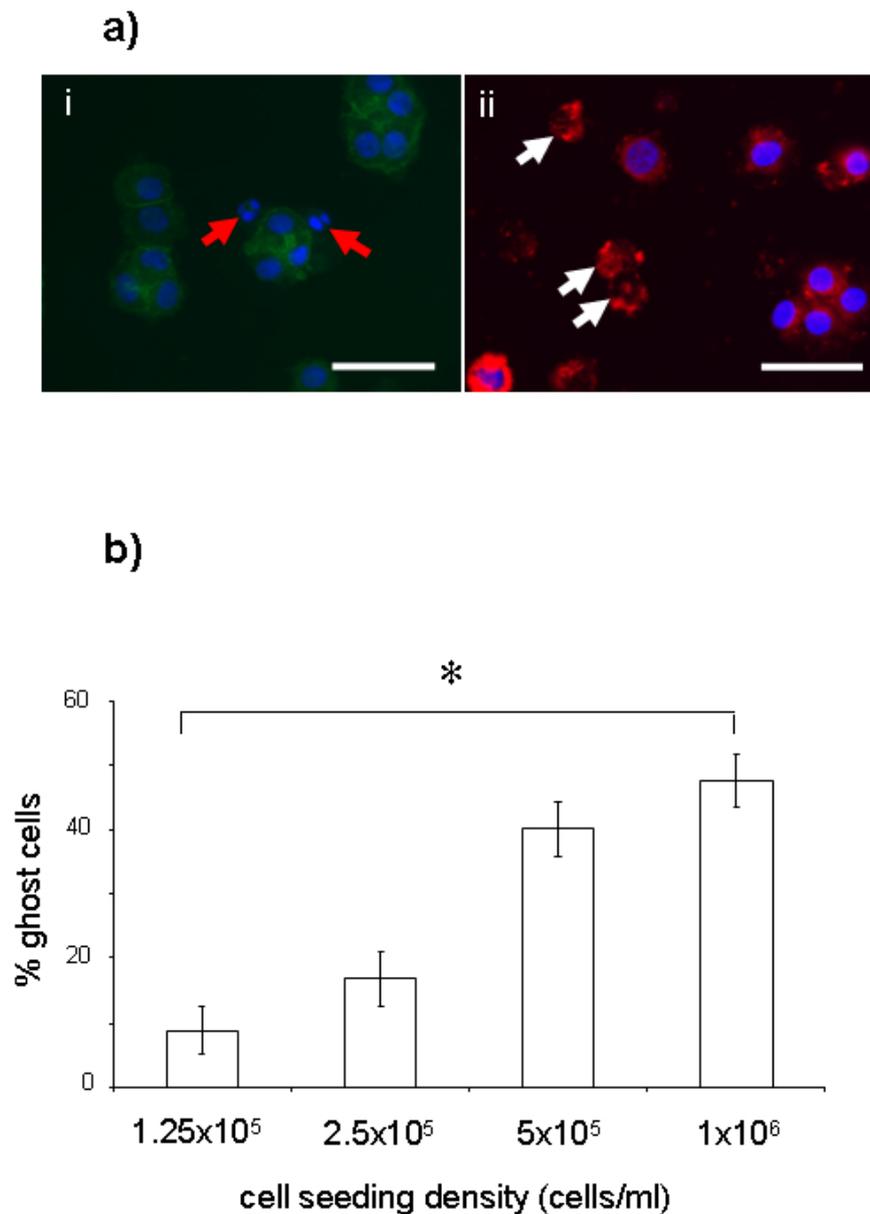
were evaluated with the Mann-Whitney  $U$  test or for more than 2 groups, with the Kruskal-Wallis ANOVA, using SPSSv15 (IBM/SPSS, Portsmouth, UK) or Analyse-It software. All data are expressed as means  $\pm$  standard deviation of the mean (SD). Significant differences were accepted at  $p < 0.05$ .

## Results

### NP cell proliferation and cluster formation were inversely related to cell density

NP cells that had been encapsulated in alginate beads were evenly distributed as single cells throughout the alginate

(day 0 cultures). In contrast, after 4 and 8 d in standard culture conditions in alginate beads, NP cells formed cell clusters, visible both *in situ* within the alginate and when harvested as cytopins. The formation of these NP cell clusters was dependent on the seeding density, such that at densities of  $1.25 \times 10^5$  and  $2.5 \times 10^5$  cells/mL, the majority of NP cells were in cell clusters, but there were fewer clusters in cultures seeded at  $5 \times 10^5$  cells/mL. At a seeding density of  $10^6$  cells/mL, most NP cells remained single, even after 8 d in culture in standard culture conditions (Fig. 1a,b). The proportion of NP cells comprising clusters at day 8 was significantly less ( $p \leq 0.05$ ) at  $10^6$  cells/mL than that at  $1.25 \times 10^5$  cells/mL. In contrast, the proportion of single NP cells was significantly greater ( $p \leq 0.05$ ) at a



**Fig 3. (a)** Images of apoptotic and “ghost” cells. **(i)** Apoptotic cells were identified morphologically with DAPI staining (blue) showing blebbed nuclei or karyorhexis (red arrows); F-actin staining (green) was greatly reduced in these cells. **(ii)** Ghost cells, consisting of cell bodies lacking DAPI-stained nuclei, were positive for the cell tracker, PKH26 red (white arrows). Scale bars represent 50  $\mu\text{m}$ . **(b)** Percentage of ghost cells (negative for DAPI staining) in cells cultured in alginate beads at standard conditions (i.e., in 10 % FCS, 17.5 mM glucose and 55 mg/L sodium pyruvate) for 8 d  $\pm$  SD (\* denotes significantly more ghost cells ( $p < 0.05$ ) in cells seeded at  $1.0 \times 10^6$  cells/mL compared to  $1.25 \times 10^5$  cells/mL).

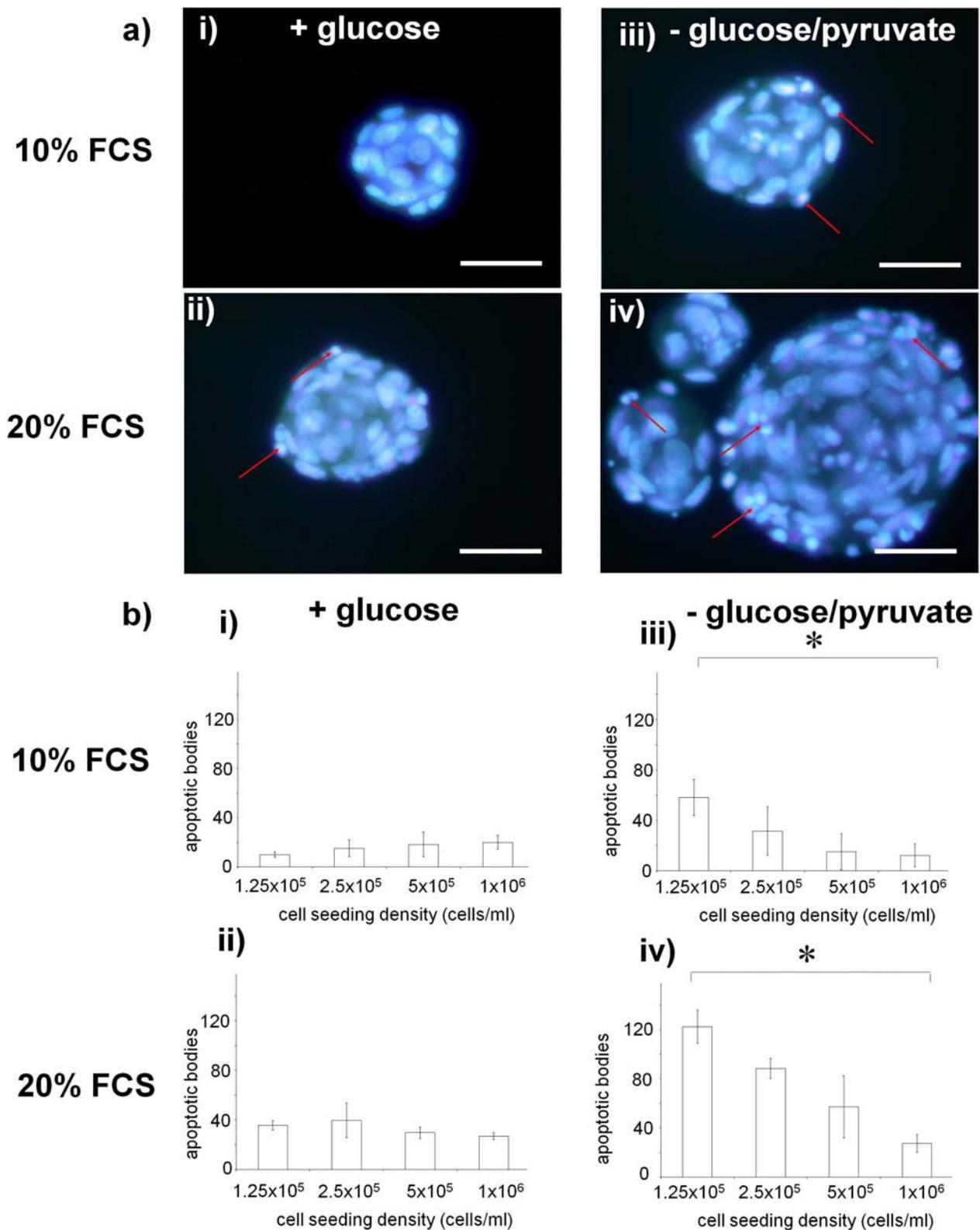
cell seeding density of  $10^6$  cells/mL than that at  $1.25 \times 10^5$  cells/mL in standard culture conditions. The number of cells in one cluster was also related to the seeding density (not significantly at day 4 ( $p = 0.74$ ), but the relationship was significant at day 8 ( $p = 0.04$ )). Hence there were more NP cells per cluster, i.e. the clusters were bigger, at a cell seeding density of  $1.25 \times 10^5$  cells/mL ( $8 \pm 1$  cells/cluster) than at a cell seeding density of  $10^6$  cells/mL in standard culture conditions ( $4 \pm 1$  cells/cluster; Fig. 1c).

Immunopositivity for the proliferation-associated Ki67 antigen was most frequently seen in NP cells within clusters in cultures seeded at  $1.25 \times 10^5$  cells/mL, where  $>70$  % of the NP cells were Ki67-immunopositive in standard culture

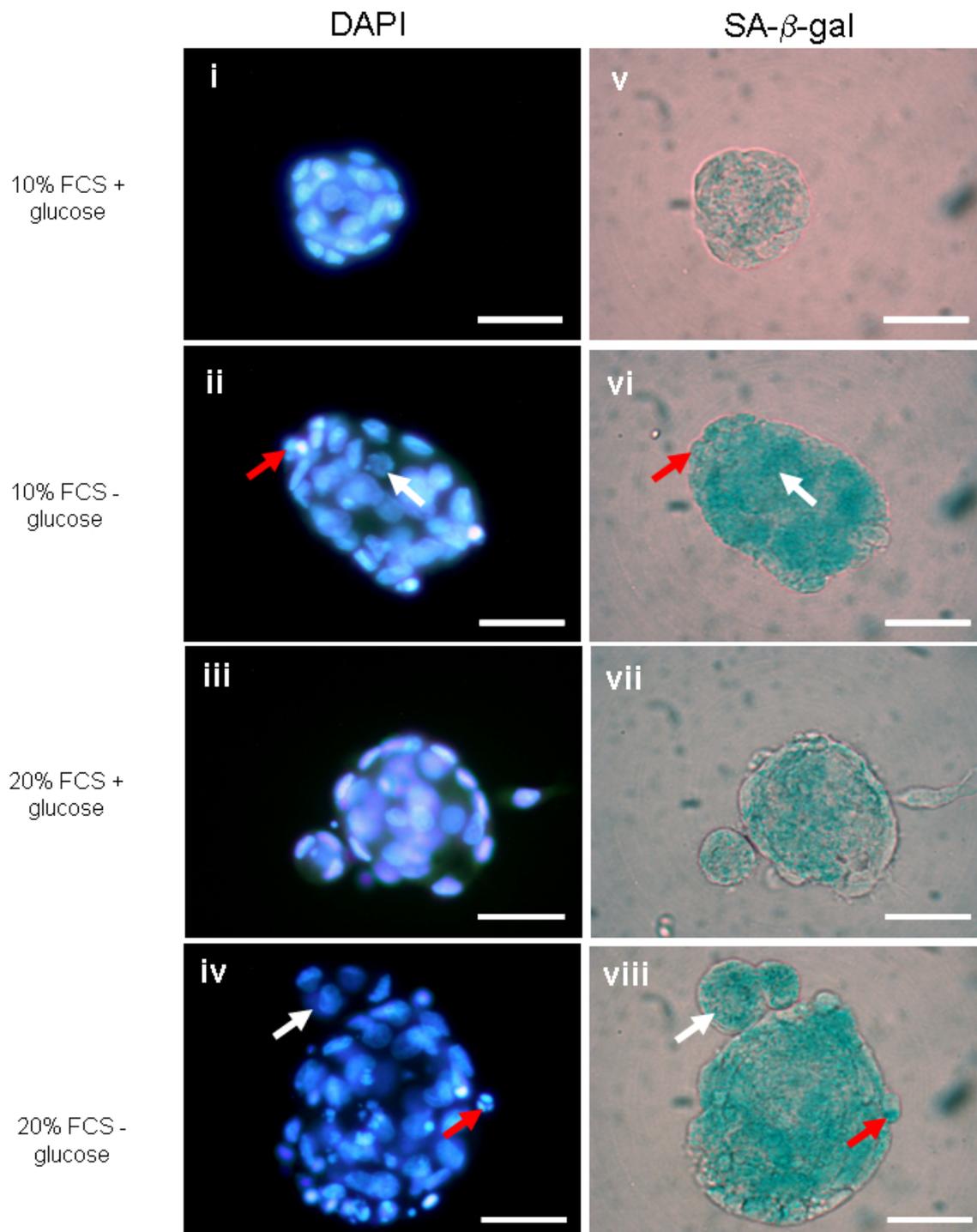
conditions (Fig. 2). Conversely, fewer NP cells seeded at the higher cell density of  $10^6$  cells/mL were Ki67-positive (with  $<10$  % cells staining positive for Ki67 antigen). There was an inverse significant relationship between Ki67 immunopositivity and seeding density ( $p = 0.02$  after 8 d in standard culture conditions in alginate beads; Fig. 2c).

#### NP cell death and “ghost cells”

The percentage of viable cells as assessed by Live/Dead staining demonstrated 90 % or more viability (staining green) for all seeding densities, with more dead cells in clusters than single cells (9 % and 1 %, respectively, in standard culture conditions) for all seeding densities except



**Fig 4.** (a) Representative images of DAPI-stained NP cells seeded into alginate beads at  $1.25 \times 10^5$  cells/mL forming clusters in (i) 10% FCS, (ii) 20% FCS, (iii) 10% FCS, without glucose or pyruvate and (iv) 20% FCS without glucose or pyruvate. Red arrows show apoptotic nuclei. Scale bars represent 50  $\mu$ m. (b) Graphs showing the frequency ( $\pm$  SD) of apoptotic cells per 200 cells when cultured in (i) 10% FCS, (ii) 20% FCS, (iii) 10% FCS without glucose or (iv) 20% FCS without glucose (\* denotes significantly more apoptotic cells in 'no glucose' conditions ( $p < 0.05$ ) at day 8 in cells seeded at  $1.25 \times 10^5$  cells/mL compared to  $1.0 \times 10^6$  cells/mL).



**Fig. 5.** Representative images of (i-iv) DAPI-stained cell clusters formed by cells seeded at  $1.25 \times 10^5$  cells/mL and (v-viii) SA-β-gal staining within the same cells showing senescence, after 8 d culture in alginate beads. (Red arrows show nuclear morphology typical of apoptotic cells in DAPI-stained images and white arrows indicate normal, viable cells.). Scale bars represent 50 μm.

$10^6$ . These results were substantiated by the counts of cells with apoptotic morphology (demonstrated by DAPI). In addition to these ‘live’ and ‘dead’ cells we also noted the presence of cell-sized entities, which did not contain DNA (as revealed by DAPI staining) but nonetheless labelled with the membrane-binding cell tracker and F-actin (Fig. 3a). The presence of these “ghost” cells was also significantly related to cell density, with most observed at

the greatest cell density when cells were grown in standard culture conditions ( $p = 0.03$  at day 8; Fig. 3b).

#### The effects of serum and glucose levels were cell density dependent

In alginate cultures maintained in 20 % FCS, the NP cell clusters that formed at low seeding densities were larger than those clusters that had formed in 10 % FCS. There

was also an apparent increase in cluster size when cultured in medium that did not contain any glucose or pyruvate, whether it was supplemented with 10 % or 20 % FCS. However, although the size of clusters in glucose-free medium was markedly larger, the number of apoptotic bodies seen also increased, though this was not statistically significant (Fig. 4a,b). The frequency of apoptosis and the size of cell clusters were greatest when cells were cultured at  $1.25 \times 10^5$  cells/mL in medium, containing 20 % FCS with no glucose or pyruvate. An increased cell seeding density was associated with decreased cell viability at day 8 in cultures maintained in 10 % FCS and in the presence of glucose. However, this inverse relationship was reversed to become a positive association between seeding cell density and cell viability in the absence of glucose, both in 10 % and 20 % FCS.

### Cell senescence

Senescent NP cells, as demonstrated by SA- $\beta$ -galactosidase staining, were commonly seen within cell clusters of cultures in both 10 % and 20 % serum with glucose. However, in those cultures that were maintained without glucose or pyruvate, the NP cells in clusters showed even more intense SA- $\beta$ -Gal positive staining (Fig. 5). This staining appeared equally intense in NP cells with normal and apoptotic morphologies.

## Discussion

An increase in the number of non-viable cells present in the human intervertebral disc has been associated with both ageing and disc degeneration (Boos *et al.*, 2002). Reduced availability of nutrients is suggested to occur through an age-related sclerosis of the cartilaginous endplates and is thought to play an important role in this pathology (Nachemson *et al.*, 1970; Boos *et al.*, 2002; Bibby and Urban, 2003; Grunhagen *et al.*, 2006;). *In vitro* experimentation has lent support to such a hypothesis, where reducing levels of glucose has been found to decrease cell viability significantly (Bibby and Urban, 2003) whilst lowering serum levels increased cell proliferation and senescence in cultured IVD cells (Johnson *et al.*, 2008). It has been suggested that modified nutrient and growth factor availability to disc cells *in vivo* may influence cell behaviour. Indeed, analysis of surgically removed human disc tissue has shown cell clusters (Ford *et al.*, 2002) composed of proliferating (Johnson *et al.*, 2001), senescent (Roberts *et al.*, 2006b) and apoptotic disc cells (Park *et al.*, 2001a).

The aim of this study was to examine further the influence of nutrient supply and the availability of blood borne growth and survival factors on IVD cells in a defined 3 dimensional system that maintains their *in vivo* phenotype (Horner *et al.*, 2002). We have also shown that NP cells form clusters in this alginate culture system, particularly when they are maintained in the absence of glucose or pyruvate (Johnson *et al.*, 2008), possibly mimicking pathological processes that occur in IVD degeneration. In that previous study (Johnson *et al.*, 2001) a constant density of 1 million IVD cells/mL was used

throughout the experimentation. In the present study, we have demonstrated that reducing the number of IVD cells below a density of  $10^6$ /mL, such as might occur in IVD degeneration, resulted in increased NP cell proliferation (as delineated both by Ki67 immunopositivity (Johnson *et al.*, 2001) and the lack of any evidence of cell migration or changes in cell morphology over time that would be associated with such migration, (e.g. the presence of cell processes or actual movement under video microscopy). Hence, we found that increased NP cell proliferation and cell cluster formation was inversely correlated with cell seeding density.

It may be that levels of both nutritive and growth or survival factors that were available to the NP cells in this culture system directly influenced their proliferative capacity. There are numerous factors present in serum that have been reported to enhance NP cell proliferation and survival, including platelet-derived growth factor (PDGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), insulin-like growth factor (IGF) and fibroblast growth factor (FGF) (Gruber *et al.*, 2000; Pratsinis and Kletsas, 2007). This perhaps explains the increased proliferation and larger clusters found with 20 % FCS compared to those with 10 % FCS. Indeed, increasing the concentration of FCS has been shown to increase proliferation rates of other cell types in culture, which has been attributed to growth factors within it (Naseer *et al.*, 2009) and the addition of FGF to wounded cartilage stimulated chondrocytes to proliferate and form clusters (Khan *et al.*, 2010).

However, in addition to evidence of cell proliferation in these culture conditions we also found evidence of cell death. Apoptotic cells, as identified by the presence of karyorhexis, were most frequent in standard culture conditions in clusters formed in cultures seeded at the highest density of  $1 \times 10^6$  cells/mL. It could be considered contradictory that apoptotic NP cells were frequently observed within clusters of cells that arose in cultures with an initial low seeding cell density, whilst apoptotic cells were also observed in cultures established at high seeding cell density. However, these observations may reflect that the level of survival factors was adequate at low seeding cell densities, until these cultures proliferated to exceed the number of cells that could be supported. The cultures with high seeding densities may reach this level at different time points. Hence, each situation might change as cell proliferation leads to the formation of cell clusters over time, such that the levels of growth/survival factors are locally limited and hence insufficient to prevent apoptosis.

Unexpectedly, however, another morphology was also found in the present study, that of cells with an intact cytoplasmic membrane but no apparent intact chromatin, or certainly none which was capable of reacting with ethidium bromide or DAPI. We have termed these "ghost cells" similar to those described in other populations of cells, for example, in odontomas, where this term describes anucleate cells with homogenous cytoplasm (Kim *et al.*, 2000). In these tumours, ghost cells can form large clusters and may be related to a malfunctioning Wnt pathway (Tanaka *et al.*, 2007) and/or apoptosis (Kim *et al.*, 2000). Certainly ghost cells were also observed in the present study to occur at significantly higher numbers in beads

seeded at  $1 \times 10^6$  cells/mL compared to those seeded at the lowest density. The formation of cells with this morphology in the present study could have relevance to disc pathology, since areas have been identified in degenerate discs resembling “cell ghosts” where remnants of cells appear to be replaced by extracellular matrix (Roberts, 2001). It is widely accepted that cells in general can die by different cellular pathways and mechanisms, and for many years disc cells have been shown to be able to die *via* necrosis or apoptosis (Trout *et al.*, 1982; Lotz and Chin, 2000). However, additional forms of cell death have been reported in other cell types including oncosis, autophagy or anoikis (Kroemer *et al.*, 2005). Indeed, recently disc cells with a different morphology, ‘balloon cells’, have been described in human discs, apparently associated with cell death (Sitte *et al.*, 2010). This suggests that we have perhaps had a more simplistic impression of the types of cell death that disc cells can undergo than is really the case.

We have previously shown that glucose/pyruvate levels can influence disc cell proliferation, both in monolayer and 3-D alginate bead culture, with them proliferating more rapidly in the absence of glucose or pyruvate than when present at 17.5 mM (Johnson *et al.*, 2001). In reality the ‘absence of glucose’ is likely to have been hypoglycaemic conditions (approximately 1–2 mM) rather than a complete absence, due to the addition of FCS in the medium which contains glucose. Elevated levels of glucose have been seen to inhibit proliferation of other cell types, e.g. endothelial cells (Chen *et al.*, 2007) or renal proximal tubule cells (25 mM glucose) (Park *et al.*, 2001b). This appears to be effected *via* oxidative stress pathways, being dependent on ERK1/2 and PKC. Hence the association with proliferation rates and levels of senescence seen in the present study is perhaps not surprising. However, survival of disc cells, as for other cell types such as heart muscle cells (Ghatnekar *et al.*, 2004) has been shown to be dependent on a minimum amount of glucose being present. Bibby and Urban (2004) demonstrated that 0.5 mM glucose is necessary for NP cell survival at 24 h in alginate culture, with cell death occurring particularly fast if grown in 21 % oxygen (rather than 0 %) or in acidic conditions. This and other studies show how important it is to study combined environmental factors which are more likely to reflect the situation *in vivo* than simply altering one variable alone, as has been demonstrated by other workers (Neidlinger-Wilke *et al.*, 2009). Our study has, however, focussed on the relationships between cell density and levels of serum and glucose only as far as cell growth and survival is concerned; clearly the capacity of the NP cells to function is of major importance. Further experimentation would be required to address ECM synthesis that is obviously very important to the functioning of the disc, particularly with respect to its mechanical properties.

The cells within the healthy intervertebral disc are normally exposed to relatively low levels of serum compared to other locations within the body. It is possible that the cells may adapt to functioning in this environment. In disorders leading to disc degeneration, for example, *via* calcification of the endplate (Benneker *et al.*, 2005) or in disc herniation when the disc’s structural integrity fails,

cells may become exposed to different levels of nutrients (Urban *et al.*, 2004) or serum and its associated growth factors. In the present study, we have shown that nutrient availability in the alginate cultures directly influences cell proliferation and cluster formation, associated senescence and apoptosis, almost to the point of suggesting that NP cells sense their ideal cell density and start to proliferate when there are few cells, or go into apoptosis if there are too many. The exposure of NP cells to additional serum (from 10 % to 20 % serum) promoted cell proliferation and formation of large cell clusters containing apoptotic cell nuclei. Lower levels of glucose/pyruvate in the culture medium, with either 10 % or 20 % serum, also had profound effects on the cellular behaviour. Hence, cells deprived of glucose/pyruvate proliferated to produce larger cell colonies, but with many apoptotic and senescent cells. (The only assessment of senescence used in this study was SA- $\beta$ -Gal positivity, which has its limitations as a technique and can fail to differentiate between cells which are senescent from those in crisis (Cristofalo, 2005)). In addition to this, senescence in a cell does not necessarily render the cell non-functional; it remains viable but may have an altered metabolism (Le Maitre *et al.*, 2007; Gruber *et al.*, 2010). This demonstration *in vitro* that the amount of serum and glucose which is available to disc cells directly influences their ability to both proliferate and survive within alginate beads could explain the presence of proliferating (Johnson *et al.*, 2001), senescent (Roberts *et al.*, 2006a), stressed (Sharp *et al.*, 2009) and/or dying cells (Roberts *et al.*, 2006b) in clusters *in vivo* in human intervertebral discs.

The influence of seeding densities in culture has been examined in other cell types, particularly with relevance to tissue engineering. The results are quite varied, however, with the cell number or density of mesenchymal stem cells being reported for example as both ‘affecting the regenerative capability’ (Serigano *et al.*, 2010) and also ‘not critical’ (Neuhuber *et al.*, 2008). Increasing the seeding density of articular chondrocytes is reported as both affecting cell behaviour (Khoshfetrat *et al.*, 2009), improving chondrogenesis (Francioli *et al.*, 2010), and as with intervertebral disc cells, leading to increased GAG and collagen production in alginate beads, but also subsequent apoptosis (Kobayashi *et al.*, 2008). Hence, in terms of cell therapy and tissue engineering of the disc, it would appear that there is likely a compromise between increasing cell number and the health status of individual cells, i.e. the simple assumption that more cells is better may not be ideal if cell expansion promotes aberrant cell behaviour and/or cell death. There is also the additional possibility that the cells may adjust themselves to the optimal population for the location by exerting autogenic control, in which case the number of cells to be introduced as cell therapy may not be critical.

## Conclusion

This study demonstrates that the optimal cell density for NP cells in alginate culture systems for tissue engineering purposes, or in the context of cell transplantation therapies, clearly depends on levels of serum and glucose

supplementation, at least in terms of cell growth and viability. As such it could have implications for both the understanding of *in vivo* degenerative disc pathology and tissue engineering methods. The proliferative capacity of disc cells may be influenced by the nutrient and/or growth factor availability *in vivo* in the disc, similar to that seen here in 3-D culture of NP cells in alginate. Cells of the intervertebral disc could experience changes in nutrient supply either in degeneration with increased calcification of the vertebral endplate (leading to decreased levels) or when there is herniation of the disc, when cells may be exposed to greater levels of glucose or serum factors which may promote localised NP cell proliferation, cellular apoptosis and senescence. We have shown that all of these biological responses occur *in vitro* and can be modified by glucose/pyruvate and serum concentrations. The exposure of NP cells to additional serum (from 10 % to 20 % serum) promoted cell proliferation and formation of large cell clusters containing apoptotic cell nuclei. Lower levels of glucose in the culture medium, with either 10 % or 20 % serum, also had profound effects on the cellular behaviour. In addition, we describe the formation of “ghost” cells, a novel morphology for the intervertebral disc. The use of NP cells in an alginate culture model hence provides further understanding of how cell biology could influence disc degeneration and also provide insight into how these processes may be manipulated to promote disc regeneration. Furthermore, this study has shown for the first time that the seeding density of NP cells may be critical to their intrinsic ability to survive and proliferate. Indeed, this study provides evidence for the existence of a possible autogenic control of NP cell number *in vivo*, which has direct relevance to potential cell therapy or tissue engineering situations.

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## Discussion with Reviewers

**Reviewer II:** What is the appropriate cell density for NP cells in alginate bead culture system based on the data? Is it somewhere between  $2.5-5 \times 10^5$ ?

**Authors:** This study demonstrates that the optimal cell density for NP cells in alginate culture systems clearly depends on levels of serum and glucose supplementation, at least in terms of cell growth and viability.

**Reviewer II:** Is there clear evidence that cell clustering seen *in vitro* culture is the same as the clusters seen in degenerate discs?

**Authors:** There is some evidence of similarity between *in vitro* and *in vivo* clusters. In both situations, cell clusters are likely to arise through clonal expansion, as indicated by the patterns of Ki-67 immunostaining (Johnson *et al.*,

2001, text reference, and the present work). In addition apoptotic cells also occur in clusters *in vivo* (Park *et al.*, 2001a, text reference).

**Reviewer II:** If so, should it be considered as mild to moderate or moderate to severe degeneration?

**Authors:** This is a difficult issue to comment on with any certainty. Pritzker (1977) (additional reference) states that in the disc these 'cell groups resembled regenerative cell nests in osteoarthritic cartilage'. We would suggest from purely personal observations that small clusters might be representative of mild to moderate degeneration whilst larger, more extensive clusters might be more typical of later stages of degeneration.

**Reviewer II:** From a cell biology point of view, when cells have the capability to proliferate, the tissue is still responding and could regenerate by itself. This is not

the assertion made by others and the authors in previous publications. Please comment about this in the context of the current study.

**Authors:** We suggest (but do not have proof) that NP cells may respond to degenerative changes in the IVD by initially proliferating to form clusters, as a part of an attempted repair process, but that this may then lead to increased cell death through apoptosis. Hence, overall there is a failure to regenerate the tissue. There are suggestions in the articular cartilage field that a similar sequence of events could occur there also.

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