DE-DIFFERENTIATION STUDY OF PORCINE NOTOCHORDAL CELLS

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ABSTRACT

Introduction. Intervertebral disc (IVD) degeneration is a worldwide health problem and causes low back pain in humans and dogs. New regenerative treatments are necessary, because current treatments are invasive, expensive, and only focus on end-stage disease. Notochordal cells (NCs) derive from the notochord and have shown to replenish and instruct the IVD cell population, acting as a tissue specific progenitor-cell and are considered to have anti-inflammatory and regenerative capacities. Because of all the challenges with regard to collecting, expanding, and culturing NCs, an alternative strategy is desirable. A promising approach could be the use of induced pluripotent stem cells (iPSCs). However, to optimize the differentiation of iPSCs to a notochordal-like cell type (NLC), the complete (epi)genetic landscape of NCs should be delineated first, by using native NCs and de-differentiated NCs as comparators.

Aim. This study aimed at generating and validating a culture system in which porcine NCs lose their specific phenotype in a controlled manner. When succeeded, these de-differentiated NCs can be used as comparators in follow up (epi)genetic analysis to delineate which specific (epi)genetic characteristics are lost and therefore typical for native NCs. In the end, this will contribute to successfully generate iPSC lines with an optimal capacity to differentiate towards mature iPS-NLCs.

Methods. Porcine NCs were subjected to different culture conditions including low glucose levels (0.006 M), high glucose levels (0.1 M), and the addition of fetal calf serum (FCS) in both a 2D (monolayer) and 3D (alginate bead) culture system. The NC morphology and phenotype, extra-cellular matrix production, and cell proliferation were determined by gene expression profiling, histological evaluation and measuring the DNA and glycosaminoglycan (GAG) content of the cultures.

Results. No distinct adverse effect of the high glucose conditions both with and without FCS was encountered with regard to the expression of different NC markers, apoptotic, and extra-cellular matrix associated genes compared to the low glucose conditions in both 2D and 3D culture systems. The DNA and GAG content of the monolayers were slightly decreased in the high glucose conditions and with the addition of FCS compared with the low glucose conditions. The DNA and GAG content of the alginate beads were increased in the high glucose conditions, whereas these were slightly decreased by the addition of FCS compared with the low glucose conditions. The cells cultured with FCS showed predominantly a spindle-shaped phenotype and a loss of vacuoles both in the monolayers as well as in the alginate beads.

Conclusions. The results of the current study suggest that FCS induces de-differentiation of NCs in both a 2D and 3D culture system, in contrast to increasing the glucose concentration. However, since the composition of FCS is uncertain and batches can vary, future research should delineate which specific growth factor can be used to induce a more controlled de-differentiation. Furthermore, because of the limitations of this pilot study, it is yet infeasible to draw definite conclusions with regard to the optimal de-differentiation culture set-up and therefore follow up studies are necessary.

INTRODUCTION

INTERVERTERBRAL DISC DEGENERATION

Intervertebral discs (IVDs) lie between adjacent vertebrae in the vertebral column. These IVDs provide mobility as well as stability to the spine and distribute pressure over the vertebrae. The IVDs consist of a nucleus pulposus (NP), annulus fibrosus (AF), transition zone (TZ), and the vertebral endplates (EPs) (Fig. 1). The notochordal cell (NC) is the main cell type of the healthy NP. NCs are found in clusters and produce an amorphous basophilic matrix rich in glycosaminoglycans (GAGs) and collagen type II. The negatively charged GAGs create a strong osmotic gradient which attracts water. The high water content results in an intradiscal pressure enabling the healthy IVD to fulfil its role as a hydraulic cushion.[1]

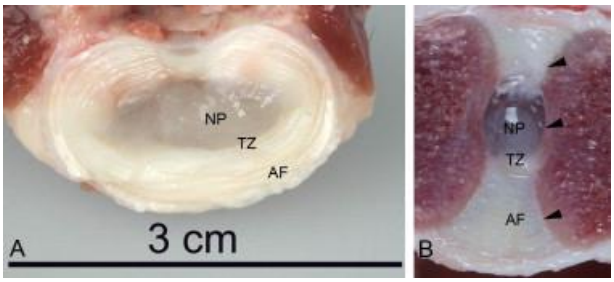

Figure 1: Images of a transverse (A) and sagittal (B) section through a healthy intervertebral disc of a mature non-chondrodystrophic dog, showing the nucleus pulposus (NP), transition zone (TZ), annulus fibrosus (AF), and endplates (arrowheads). Reprinted from Bergknut *et al.* (2013).[1]

IVD degeneration is a worldwide health problem in both humans and dogs. Together with herniation it is considered as the main cause of acute and chronic low back pain.[1] Since IVD degeneration develops spontaneously in both species and they show similarities in IVD alterations and pathology patterns, it has been established that the dog can serve as a suitable model for IVD degeneration in humans.[2] During IVD degeneration, the biochemical composition and mechanical integrity of the IVD changes. The early stage of degeneration is characterized by cellular changes within the NP. The large NC clusters are lost, resulting in smaller clusters or single NCs (Fig. 2). In essence, NCs are replaced by chondrocyte-like cells (CLCs) and their associated extracellular matrix (ECM), which resembles hyaline cartilage and consists of disorganized collagen fibers. This process is referred to as chondrification. Due to the overall loss of GAGs and the replacement of long chondroitin sulphate side chains by shorter keratin sulphate side chains, the osmotic gradient decreases, which results in less water attraction. Furthermore, a disbalance between anabolic and catabolic activity occurs, which finally results in a loss of ECM. Matrix repair is impaired in the avascular IVD, resulting in weakening and increased vulnerability to damage by physiologic loading. Altogether, the IVD can no longer properly fulfill its role as shock absorber and diffusion of nutrients becomes impaired. This suboptimal biomechanical function and lack of nutrients cause a vicious circle which further deteriorates the state of degeneration. All these aforementioned factors could eventually lead to clinical signs known as IVD disease.[1,3]

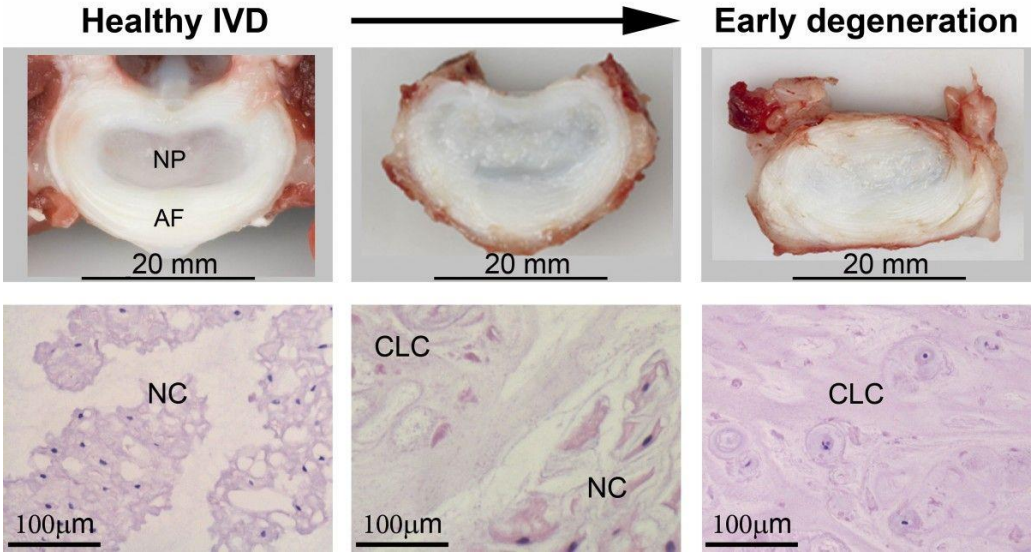

Figure 2: The macroscopic and microscopic changes with regard to intervertebral disc (IVD) degeneration. The images on the left show a healthy IVD, the middle ones the transition phase and the right images early IVD degeneration. The upper pictures show the transition from a well-distinguished lamellar annulus fibrosus (AF) and a bean-shaped nucleus pulposus (NP) (left) to less clear distinction between AF and NP (middle and right). The lower images show that the main cell type present in the healthy NP are notochordal cells (NCs) (left), whereas in the transitional NP, a mixture of cartilage-like cells (CLCs) and NCs is present (middle). In the early degenerated disc, CLCs are the main cell type present in the NP (right). Reprinted from Bach *et al.* (2014).[4]

CURRENT & NEW THERAPIES

Currently, no therapy is available that has the potential to cure the degenerated IVD. Consequently, current treatments are restricted to treating the symptoms of the disease. These treatments could be either surgical or non-surgical. Patients suffering from IVD degeneration can undergo surgical procedures that are based on decompression. Thus, these surgeries aim at alleviating the compression of neural structures which could be present as a result of herniation of the NP. Such procedures include for example nucleotomy, laminectomy, and a ventral slot procedure. Examples of non-surgical therapies are alteration of exercise patterns, weight-loss, and pain-relieving drugs such as corticosteroids and non-steroidal anti-inflammatory drugs. Since none of these treatments take away the cause of the disease but only reduce the pain and/or retard the degenerative process, current therapies could be classified as palliative care.[4]

A more effective approach to treat IVD-disease would be to actually repair and rejuvenate the degenerated IVDs. To accomplish this, it would be beneficial to tackle this degenerative disorder in the initial phase by correcting the altered cell-population. For this reason there is increasing interest in developing new regenerative treatments. Regenerative strategies aiming at treating orthopedic disorders predominantly focus on combining various cell types with certain growth factors. Strategies that increase cell numbers are necessary because relatively few cells remain in degenerated IVDs and the viability of these remaining cells is impaired. For this reason, stimulation of only the remaining cells may be insufficient to attain adequate repair.[5] Potential candidates for cell-based therapies include CLCs, mesenchymal stromal cells (MSCs), NP progenitor cells, and NCs. Several studies have shown that the insertion of autologous and allogenic CLCs in the IVD retard IVD-degeneration in different species.[6,7] However, the regenerative potential of the CLCs can be limited depending on the severity of IVD degeneration. Furthermore, CLC transplantation is hampered by the low cell numbers available.[4] To overcome this problem, there is an increasing interest in strategies that involve stem cells. For example, the use of MSCs could provide the solution. These cells are multipotent stem cells and are characterized by the ability of self-renewal and differentiation into various cells, including chondrocytes, osteoblasts, and adipocytes. Furthermore, it is suggested that these cells have profound potential to support IVD regeneration because of their immunosuppressive properties and the ability to secrete trophic factors.[8] However, there is uncertainty with regard to the chondrogenic differentiation of MSCs despite the promising experimental studies and clinical trials.[4] Recently, Tie2/TEK receptor tyrosine kinase positive NP progenitor cells were identified in bovine, canine, mouse, and human specimens.[9] These cells show remarkable multilineage differentiation capacity and direct correlation with IVD degeneration and are therefore an interesting target for regenerative strategies.[10] It was reported that, despite the relatively low Tie2+ cell numbers obtainable from degenerated IVDs from patients, proliferation rate, lineage differentiation potency, and regenerative capacity was retained.[11] Interestingly, these Tie2+ cells had a superior differentiation capacity towards the chondrogenic lineage in comparison with bone marrow-derived MSCs collected from the same patients.[11] However, Tie2+ cells are not available in the required cell quantities for clinical applications. Furthermore, the proportion of Tie2+ cells is rapidly lost during expansion.[12] Yet another promising cell type for the use of cell based therapy is the NC which is, as stated above, the main cell type of the healthy NP. Since the current study focusses on this specific cell type their specific characteristics will be discussed in more detail below.

CHARACTERISTICS OF NCs

NCs derive from the notochord which forms the NP during embryonic development. NCs have a very specific morphology: they are large round cells and obtain vacuoles of which the function is not yet delineated.[4] These cells are often present in clusters and produce collagen type II and proteoglycan rich matrix.[4] Furthermore, recent studies show that NCs secrete factors that are essential for IVD development, and stimulate mature CLCs to produce a healthy matrix.[13-15] They replenish and instruct the NP cell population, acting as a tissue specific progenitor cell and are considered to have anti-inflammatory and regenerative properties.[13-15] It is reported that in humans and certain dog breeds, cells with NC morphology disappear even before skeletal maturity is reached.[7-9] Since in these species onset of IVD degeneration is early, it is suggested that NCs play a vital role in IVD homeostasis. Therefore it seems a promising approach to repopulate IVD tissue with regenerative NCs to restore the biomechanical function of the IVD.[16,17]

However, there are some limitations with regard to applying this NC based strategy. Firstly, primary NCs are not available for human and canine applications in the required cell quantities for the large patient populations. Secondly, the expansion and culture of NCs is hampered by the fact that they lose their vacuolated phenotype and NC-specific markers. Several studies have been performed aiming at optimizing the expansion and culture conditions for NCs. The results of the study of Omlor *et al.* (2014) showed that NCs remained significantly larger throughout the cell culture experiment (16 days) and retained their vacuoles to a higher degree when cultured in hypoxic conditions (1% O_2).[18] The study of Spillekom *et al.* (2014) demonstrated that increasing the culture medium osmolarity from 300 to 400 mOsm/L and the use of α-MEM is beneficial effects on preserving the NC phenotype in culture.[19] The authors suggested that since IVD cells are subject to low glucose concentrations *in vivo*,[20] the relatively low glucose content in a-MEM may facilitate NCs to retain their phenotype.[19] Furthermore, hyperglycemia has been positively correlated with IVD degeneration in diabetic rats[21] and it has been shown that a high glucose environment stimulates apoptosis and inhibits proliferation of NCs *in vitro*.[22] The results of the study of Erwin *et al.* (2009) revealed that NCs thrive best in three-dimensional culture under hypoxic conditions (3.5% O_2).[23] The group of Humphreys *et al.* (2018) defined an optimized system for culturing NC cells by incorporating laminin-521-coated surfaces, the use of α-MEM media adjusted to 400 mOsm/L and hypoxic conditions (2% O_2). Their study also indicated that substrate stiffness was an essential consideration, with soft substrates promoting retention of phenotype.[24] The study performed by Smolders *et al.* (2012) showed that gene expression of *brachyury* and *cytokeratin 8* was significantly decreased at all time points in monolayer culture compared with NC-rich NP tissue, indicating a loss of notochordal phenotype and potential de-differentiation of NCs when cultured in monolayers.[25] Additionally, the study from Gantenbein *et al.* (2014) provided evidence by flow cytometry that monolayer culture is not favorable for NC culture with respect to retaining the NC phenotype.[26] Lastly, the study of Arkesteijn *et al.* (2016) showed that the NC phenotype was better retained in serum-free media, which could be an argument against the use of fetal calf serum (FCS) in culture.[27]

Because of all the challenges with regard to collecting, expanding and culturing NCs, an alternative strategy is desirable. A promising approach could be the use of induced pluripotent stem cells (iPSCs) which could be derived from any somatic cell type and possess unlimited self-renewal and differentiation potential.[28] To optimize the differentiation of iPSCs to a notochordal-like cell type (NLC), (epi)genetical modification of the iPSCs could be beneficial. To discover which (epi)genetic modifications would be useful to mimic the native mature NCs, it is necessary to first delineate the complete (epi)genetic landscape of NCs and use de-differentiated NCs as comparators.

AIM & APPROACH

This pilot study aimed at generating and validating a culture system in which porcine NCs lose their specific phenotype in a controlled manner. When succeeded, these de-differentiated NCs can be used as comparators in follow up (epi)genetic analysis to delineate which specific (epi)genetic characteristics are lost and therefore typical for native NCs. In the end, this will contribute to successfully generate iPSC lines with an optimal capacity to differentiate towards mature iPS-NLCs.

Based upon the results of aforementioned studies with regard to the effect of different culture conditions on the phenotype of NCs, for the present study it was chosen to induce de-differentiation of the NCs by increasing the glucose level from 0.006 to 0.1 M and subject the cells to 10% FCS. Additionally, this effect was tested in both a 2D (monolayer) as 3D (alginate bead) culture system. The NC morphology and phenotype, extra-cellular matrix production, and cell proliferation were determined by gene expression profiling, histological evaluation and measuring the DNA and GAG content. It was hypothesized that, comparable with previously conducted studies, high glucose conditions would stimulate apoptosis, inhibit proliferation, and induce loss of phenotype of NCs *in vitro*.[19,22] Furthermore, it was hypothesized that the addition of FCS would reinforce this effect since is it suggested that the NC phenotype is better retained in serum-free media.[27] Lastly, it was expected that the NCs would lose their specific phenotype to a bigger extent in the 2D compared to the 3D culture system.[23,25,26]

In this pilot study, porcine NP tissue was used since it is easy accessible and consists predominantly of NCs.[29]

MATERIALS & METHODS

HARVEST & ISOLATION OF THE NCS

The cervical and thoracic part of two porcine spines (6 weeks of age) were collected from piglets that had been euthanized for an unrelated research study (AVD7210020185884; approved by the Utrecht University Animal Ethics Committee). The pig spines were washed according to the surgical protocol (2 times chloorhexidine-gluconaat 40 mg/ml) and sprayed with chlorhexidine 2 times. The IVD-spaces were opened with a sterile blade number 22 and distracted with a Hohmann retractor. The gel-like NP was collected and put in α-MEM (22561; Invitrogen) with 1 % P/S (P11-010, GE Healthcare Life Sciences, Eindhoven, the Netherlands).

Next, the NP tissue was transferred to a clean conical 50 mL tube and centrifuged at 500 *g* for 5 minutes. After removing the supernatant, the complete sample was digested using a 0.1 % pronase (11459643001, Roche Diagnostics, IN, USA) solution in α-MEM with 1 % P/S during 30 minutes at 37ºC on a shaker. Thereafter, α-MEM with 1 % P/S was added to dilute the pronase. Next, the supernatant was removed and a 0.1 % collagenase II (4176, Worthington, Lakewood, NJ, USA) solution in α-MEM with 1 % P/S was added. The tissue was digested overnight at 37 ºC on a shaker. The next day the sample was centrifuged and half of the sample was subjected to a second digestion step by using 20 units/mL elastase (source unknown) for one hour at 37ºC. Next, the NCs were filtered over a 40-µm cell strainer (BD Biosciences). The cell clusters were collected by washing the filter with α-MEM with 1 % P/S. The sample was centrifuged at 500 *g* for 5 minutes whereafter it was resuspended in 5 mL α-MEM with 1 % P/S. The cells were counted using an automatic cell counter (TC20™, Bio-Rad). Freshly isolated cells were collected on a slide using a Shandon Cytospin 4 to assess the NC morphology before starting the culture. Furthermore, cells were collected for gene expression profiling at day 0 of the culture.

MONOLAYER CULTURE

The NCs digested with collagenase II alone and with both collagenase and elastase were plated separately in either 48-well plates (677180, CELLSTAR® Greiner Bio-one, Alphen a/d Rijn, the Netherlands) or glass 8-well chamber slides (Millicell EZ SLIDE 8-well glass; PEZGS0816, Sigma-Aldrich) at a cell density of $2 \cdot 10^4$ cells/well (based upon the available amount of cells) in 250 µL α-MEM with 1% P/S, 1% ITS+ premix (354352, Corning Life Sciences, Amsterdam, the Netherlands), and 1.25 mg/mL Bovine Serum Albumin (Sigma Aldrich, A9418). To increase the osmolarity of the most optimal culture condition from 300 to 400 mOsm/mL, 1% 5M NaCl (31434; Sigma-Aldrich) and 1% 0.4M KCl (100737; Merck) were added. To induce de-differentiation, 10% 1M D(+)-glucose[22] (100511; Merck) alone or together with 10% FCS[27] (high performance 16000–044, Gibco, Bleiswijk, The Netherlands) was added to the medium (Table 1). The cells were put in a humidified air incubator at 37ºC under hypoxic conditions (5% O_2). Because the NC clusters cultured in the serum-free media did not adhere to the plate up until day 7, it was decided to apply a surface coating. Since Humphreys *et al.* (2018) reported the most optimal cell adherence using a laminin coating the clusters were transferred to culture surfaces modified through overnight incubation on a shaker at room temperature with 110 µL per well of 20 µg/mL laminin-521 (Appleton Woods, Birmingham, UK) in PBS at day 7.[24] Wells were washed with 1 mL PBS before seeding the clusters again. Because of a limited amount of laminin available, the clusters digested with collagenase alone and with both collagenase and elastase cultured in the same conditions were pooled resulting in $4 \cdot 10^4$ cells/well (Fig. 3). The cells were cultured for another 7 days. The NCs cultured with FCS using the two different digestion methods were pooled at the end of the culture period (day 14) (Fig. 3).

Table 1: Composition of the different culture media

Experimental Group	Composition	Osmolarity (mOsm/L)
Low glucose	a-MEM + 1% 5M NaCl (31434; Sigma-Aldrich) + 1% 0.4M KCl (100737; Merck)	400
High glucose	a-MEM + 10% 1M D(+)-glucose (100511; Merck)	400
High glucose + FCS	a-MEM + 10% 1M D(+)-glucose + 10% FCS	400

a-MEM: a-minimal essential medium (22561; Invitrogen) + 1% penicillin/streptomycin (P11-010, GE Healthcare Life Sciences, Eindhoven, the Netherlands) + 1.25 mg/mL Bovine Serum Albumin (Sigma Aldrich, A9418); FCS: fetal calf serum (high performance 16000–044, Gibco, Bleiswijk, The Netherlands).

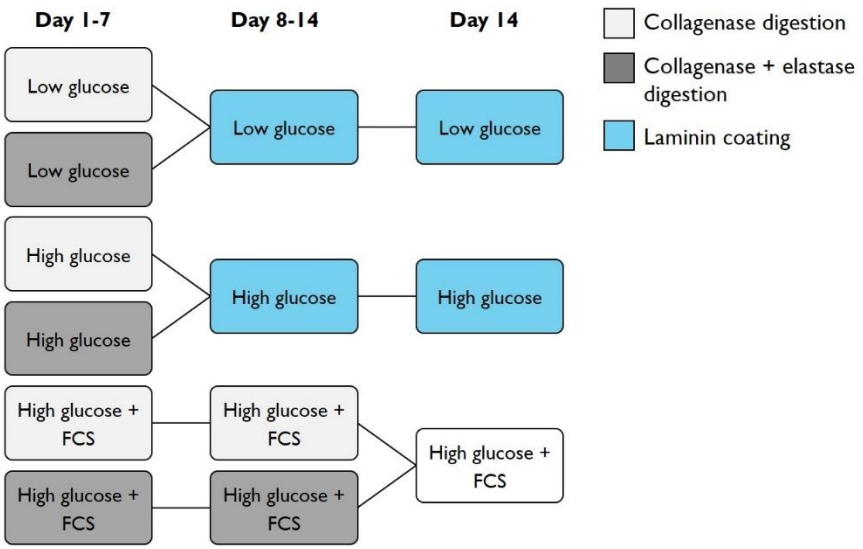

Figure 3: Overview of the monolayer culture period. Two different digestion methods were used: 0.1% collagenase II alone (light grey) or followed by 20 units/mL elastase (dark grey). Three different experimental groups were created: Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + fetal calf serum (FCS): α-MEM + 0.1 M D-glucose + 10% FCS. After 7 days of culture the samples subjected to the different digestion of the serum-free groups were pooled and transferred to surfaces coated with 20 µg/mL laminin-521 (blue). At the end of the culture (day 14) the samples cultured with FCS were pooled as well.

ALGINATE BEAD CULTURE

NCs digested with only collagenase II were encapsulated in alginate beads by adding 2.4% sterilized alginate solution (A2033; Sigma-Aldrich) 1:1 (v/v) to the cell suspension, with a final cell density of $4 \cdot 10^5$ cells/mL (based upon the available amount of cells). The alginate/cell suspension was then gently pressed through a 26-gauge needle into a sterilized solution of 102 mM CaCl2 (50380; Boom) and 10 mM HEPES (H4034; Sigma-Aldrich) to form solid beads. The alginate beads were cultured in 24-well plates (662160, CELLSTAR® Greiner Bio-one, Alphen a/d Rijn, the Netherlands)) in a humidified air incubator at 37°C under hypoxic conditions (5% O_2), divided into the same experimental groups as the monolayers (Table 1). The alginate beads (5 beads/well) were cultured in 700 µL culture medium for the entire culture period of 14 days. The medium was replaced two times/week and the beads were treated with calcium chloride (102 mM CaCl2 + 10 mM HEPES) at day 7 to ensure the beads stayed intact.

GENE EXPRESSION PROFILING

RNA ISOLATION

RNA isolation of the tissues used for testing new designed primers was performed using the RNeasy® Mini Kit (74106, Qiagen, Valencia, USA) whereas the RNeasy® Micro Kit (74004, Qiagen, Valencia, USA) was used for isolating the RNA of the cultured monolayers and alginate beads. Firstly, buffers and DNase solution were prepared as described for RNA isolation of the tissues and culture samples. Ten µL β-mercaptoethanol (β-ME) was added per 1 mL Buffer RLT to effectively inactivate RNAses in the lysate. Four volumes of 96% ethanol were added to Buffer RPE to create the working solution. The DNase I stock-solution (RNAse-Free DNase Set, 79254, Qiagen, Valencia, USA) was prepared by dissolving lyophilized DNase I in 550 µL RNase free water.

The tissues were first frozen in liquid nitrogen and pulverized with a hammer. This was repeated two more times whereafter 600 µl buffer RLT+β-ME was added. To the monolayers, 350 µl buffer RLT+β-ME was added per well. The sample was lysed and homogenized by pipetting up and down whereafter the well surfaces were scraped and the samples collected in vials. The alginate beads were collected and put in vials (5 beads/well). Sodium citrate buffer was prepared containing 55 mM tri-natriumcitraat-2-hydrate (Merck 6448), 0.15M sodium chloride (Merck 6404), and 25mM HEPES (Sigma H4034) in RNase free water. The pH was adjusted to 7.4. After removing the

medium, 1 mL sodium citrate buffer was added per well. After 5 minutes of incubation, the sample was mixed and centrifuged for 5 minutes at 500 *g*. The supernatant was removed and 300 µL buffer RLT+β-ME was added.

To all samples an equal volume of 70% ethanol was added to precipitate the RNA and the solution was mixed well by pipetting. Next, the samples were transferred to a RNeasy MinElute spin-column in 2 mL collection tubes, and centrifuged at 16100 *g* for 30 seconds to separate the precipitated RNA from the solution. Thereafter, the flow-through was discarded and 350 µL Buffer RW1 was added to the RNeasy MinElute spin columns to remove non-specifically bound biomolecules to the silica-membrane. Then the samples were centrifuged for 30 seconds at 16100 *g*, whereafter the flow-through was discarded. The DNase I incubation mix was prepared by adding 45 µL DNase I stock solution to 315 µL Buffer RDD. Next, the DNase I mix (80 µL) was pipetted directly to the RNeasy MinElute spin columns membrane, and incubated for 15 minutes to digest the genomic DNA. Next, 350 µL Buffer RW1 was added to the RNeasy MinElute spin columns again, whereafter the columns were centrifuged for 30 seconds at 16100 *g*. The RNeasy MinElute spin columns were placed in new 2 mL collection tubes (supplied), whereafter 500 µL Buffer RPE was added to the spin columns for washing the membrane-bound RNA. The samples were centrifuged for 30 seconds at 16100 *g*, whereafter the flow-through was discarded. To the tissue samples again 500 µL Buffer RPE was added to the spin columns whereas to the monolayer- and alginate bead-samples 500 µL 80% ethanol was added. The samples were centrifuged for 2 minutes after which the RNeasy MinElute spin columns were placed in new 2 mL collection tubes. After opening the lids, the samples were centrifuged at full speed for either 1 minute (tissue samples) or 5 minutes (monolayer- and alginate bead-samples) to dry the membrane. The flow-through was discarded and collection tubes were discarded and the RNeasy MinElute spin columns were placed in new 1.5 mL collection tubes. Either 32 µL (tissue samples) or 17 µL (monolayer- and alginate bead-samples) RNase-free water was added directly to the center of the spin columns membrane after which the samples were centrifuged for 1 minute at 16100 *g* to elute the RNA. The flow-through was added directly to the center of the spin columns membrane again and for the last time, the samples were centrifuged at full speed for 1 minute. The spin columns were discarded and after the quantity of RNA was determined by using a spectrophotometer (DeNovix DS-11) the samples were stored at -80ºC.

cDNA SYNTHESIS

The iScript™cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands) was used to synthesize cDNA. 4 µL of iScript Reaction Mix and 1 µL of iScript Reverse Transcriptase were added to 15 µL of RNA resulting in a final volume of 20 µL per vial. The complete reaction-mix was incubated for 5 minutes at 25 ºC, 30 minutes at 42 ºC, and 5 minutes at 85 ºC. The samples were stored at -20 ºC until further use.

QUANTITATIVE RT-PCR

Since there was a limited amount of primers readily available for analyzing the porcine NCs, lacking primers were designed using PerlPrimer 1.1.21. The new primers were first tested for the optimal temperature by running a gradient on cDNA of tissues in which the genes were highly expressed according to the human protein atlas version 18.1.

Three readily available reference genes (Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), and Ribosomal Protein S19 (RPS19)) were used to normalize gene expression of the target genes. Target genes included several NC markers, matrix production-associated, and apoptosis genes (Table 2).

TABLE 2: REFERENCE AND TARGET GENES

Gene name	Gene symbol	Forward primer sequence	Reverse primer sequence	Temp
NC markers				
Cytokeratin 8	KRT8	GAGGACTTCAAGAACAAGTATGAG	AGTTGATCTCGTCGGTCAG	61.5
Cytokeratin 18	KRT18	AAGACTATCGAGGAACTGAGG	TGAAGTCATCAGCAGCGA	57
Cytokeratin 19	KRT19	AACCACGAGGAGGAAATCAG	CTCATACTGGCTTCTCATGTC	64
Cluster of differentiation 44	CD44	ATGACGAAGAGGAGACCCAG	CATCCATTCTCAAACCACTGCT	67
Carbonic anhydrase	CA	ATTGAACTGCACACTAAGGAC	CTCTCAGCATTGACCTGTC	61.5
Noggin	NOG	TGCCGAGCGAGATCAAAGGG	AGCCACATCTGTAACTTCCTCCG	64
Forkhead box protein A1	FOXA1	GTTAGGGACTGTGAAGATGG	CGAGTTCATGTTGCTGACC	58
Matrix production-associated genes				
Collagen type I a1	COL1A1	GAGATGCTGGTCCCAAAGG	CGCTAGGACCAGTTTCACC	66
Aggrecan	ACAN	CAACTACCCGGCCATCC	GATGGCTCTGTAATGGAACAC	63.5
A disintegrin and metalloproteinase with thrombospondin motif 5	ADAMTS 5	GATTGAAGATGATGGCCTCC	CGCTTATCTTCTGTGGAACC	62.5
Matrix metalloproteinase 13	MMP13	TCCCAGGAATTGGTGATAAAGTAGA	CTGGCATGACGCGAACAATA	64
SRY-box 9	SOX9	CCCAACGCCATCTTCAAGG	CTGCTCAGCTCGCCGATGT	65.5
Apoptosis genes				
Bcl-2-associated X	BAX	GGACGAACTGGACAGTAACATGG	GCAAAGTAGAAAAGGGCGACAAC	58
B-cell lymphoma 2	BCL2	ATCGCCCTGTGGATGACTGAG	CAGCCAGGAGAAATCAAACAGAGG	64
Caspase-3	CASP3	GAACAAATGGACCTGTTGATCTG	AGTCCAATTCTGTGCCTCG	57
References genes				
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	CTGCCCAGAACATCATCCC	CAGTGAGCTTCCCGTTGAG	58
monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	CAAAGACAGCATTTGATGAAGCC	ATCTCCTTGGGTATCCGATGTC	65
Hydroxymethylbilane synthase	HMBS	GATGGGCAACTCTACCTGAC	CAAGCTGTGGGTCATCCTC	67
Ribosomal Protein S19	RPS19	AAAGAAACGGTGTCATGCCC	AGGCCTTTCCCATCTTGGT	62

Quantitative RT-PCR was performed using the iQT™ SYBR Green Supermix-Kit (Bio-Rad, Veenendaal, the Netherlands) and the CFX384 Touch™ Real-Time PCR Detection-System (Bio-Rad, Veenendaal, the Netherlands). To show that the PCR reaction was working optimally an internal standard was used (efficiency is 95-105 %). Furthermore, the internal standard shows the detection limit of the PCR reaction. The standard samples ranged from 1 until 7 and consisted of a serial 4 times dilution of a pool of 10 times diluted cDNA from all the samples. To obtain 50 times diluted cDNA the 10 times diluted cDNA was diluted another 5 times. A master-mix was prepared containing SYBR Green, forward, and reverse primer in concentrations adjusted to the experiment. 6 µL master mix and 4 µL 50 times diluted cDNA were pipetted in 384 wells plates according to the designed plate set-up. The plates were centrifuged for 1 minute at 593 *g* before they were put in the PCR machine at the right temperature and protocol (2- or 3-steps).

To obtain the relative quantitative gene expression the Normfirst ($E^{\Delta\Delta Cq}$) method was used. The Cq-value of the test sample and the calibrator sample for each target gene was normalized to the mean Cq-value of the reference genes, using the following formula: $\Delta Cq = Cq_{mean\ ref} - Cq_{target}$. As calibrator the Cq-values of the samples collected directly after digestion (day 0) were used. Next, the $E^{\Delta Cq}$-values for the test and calibrator samples were

calculated. 'E' indicates the amplification efficiency of the target- or reference-gene in this formula. Thereafter, $E^{\Delta\Delta Cq}$ was calculated by normalizing the EΔCq-value of the test-sample to the value of the calibrator: $E^{\Delta\Delta Cq} = E^{\Delta Cq\ test} - E^{\Delta Cq\ calibrator}$. Lastly, the mean n-fold change and standard deviation in gene expression were calculated for each target-gene. Gene expression of the samples collected at day 0 was set at 1.

GAG & DNA MEASUREMENTS

In order to measure the GAG and DNA content of the monolayers and alginate beads, they were first digested. For digesting the monolayer samples 100 µL of papain digestion solution (pH 6, 200 mM H_2 $NaPO_4$ *2 H_2O (21254, Boom B.V., Meppel, the Netherlands)), 10 mM EDTA (100944, Merck Millipore, Amsterdam, the Netherlands), 10 mM cysteine HCl (C7880, Sigma Aldrich, Saint-Louis, USA), and 10 mM papain (P3125, Sigma Aldrich, Saint-Louis, USA)) was added to each well. After 1 hour of incubation at 60 °C, the wells were scratched and the samples were collected and transferred to vials. For digestion of the alginate beads, three beads were collected per vial and after removing the medium put in 100 µL of papain digestion solution. All samples were incubated overnight at 60 °C. The next day, the samples were vortexed whereafter they were incubated for another hour at 60 °C.

During the second week of the culture each medium change, 100 µL culture medium of each well, used for GAG and DNA measurements, was collected and pooled. Before quantification of the GAG content by the dimethyl methylene blue (DMMB) assay, the digested monolayers and the collected medium of the monolayers were diluted ten times. The alginate beads were diluted 25 times and the collected medium of the beads 5 times. Directly after DMMB (341088, SigmaAldrich, Saint Louis, USA) was added, the absorbance (540/595 nm) was determined using a microplate reader. To avoid any electrostatic interference with the colorimetric assay, alginate had been removed from the sample by means of acid precipitation, adjusting the pH to 1.5. The GAG content was calculated using a chondroitin sulphate (C4384, Sigma-Aldrich, Saint Louis, USA) standard line with polynomic properties.

The DNA content per micro-aggregate and three alginate beads was measured using the Qubit® dsDNA High Sensitivity Assay Kit (Q32851, Invitrogen, Paisley, UK). The Qubit™ reagent was diluted 1:200 in Qubit™ buffer to prepare the Qubit™ working solution. The standard assay tubes were prepared by the addition of 10 µL standard from the kit to 190 µL of Qubit™ working solution. The sample assay tubes were prepared by adding 10 µL of sample to 190 µL of working solution. All tubes were vortexed for 2-3 seconds. The standard line was determined by measuring the standard assay tubes, whereafter the assay tubes were inserted in Qubit® 2.0 and readings were taken.

HISTOLOGY

At day 14, the monolayers on the chamber slides were fixed in 4% neutral buffered formaldehyde (4286, Klinipath B.V., Duiven, the Netherlands) for 24 hours at room temperature (RT). The alginate beads were put in 4% neutral buffered formaldehyde with 10% eosin for 24 hours at RT. The eosin was used to improve the visibility of the alginate beads within the paraffin blocks. Next, the alginate beads were embedded in 2.4% alginate (Sigma Aldrich, A2033) which was cross-linked by adding formalin with 100 mM $CaCl_2$. The samples were stored in embedding cassettes in 70% alcohol until they were dehydrated through graded alcohol steps. After dehydration, samples were embedded in paraffin and 5 µm sections were mounted on Microscope KP+ slides (KP-3056, Klinipath B.V., Duiven, The Netherlands).

HEMATOXYLIN AND EOSIN STAINING

For the hematoxylin and eosin (H&E) staining, the alginate bead sections were deparaffinized through xylene (two times 2 minutes) and graded ethanol (100% two times 1 minute; 96% and 50% for 1 minute), followed by one PBS rinse. Subsequently the alginate beads were treated with 10 mM citrate buffer (pH 6) to dissolve the alginate. All slides were subjected to Mayer's haematoxylin (3870, J.T.Baker® Chemicals - Avantor Performance Materials, Center Valley, USA) for 4 minutes and rinsed with water for 5 minutes. Thereafter the slides were put in

hydrochloric acid fuming 37% (1.00317.100, Merck) diluted 400 times in 50% ethanol for 15 seconds. After 1 minute in 70% ethanol the slides were subjected to 1.5% ammonia solution (1.05426.1000m Merck) for 15 seconds. After 1 minute in 96% ethanol the slides were stained with Eosin Y (C.I. 45380, Sigma Aldrich). The slides were then dehydrated in 100% ethanol two times 1 minute and xylene two times 1 minute and mounted using Pertex mounting medium (LEIC811, LEICA MICROSYSTEMS).

SAFRANIN O/FAST GREEN STAINING

For the Safranin O/Fast Green staining, the alginate bead sections were deparaffinized through xylene (two times 5 minutes) and graded ethanol (100% two times 3 minutes; 96% and 70% both two times 1 minute), followed by one PBS rinse. Subsequently, the alginate bead slides were treated with 10 mM citrate buffer (pH 6) for 15 minutes to dissolve the alginate. Thereafter all slides were subjected to Mayer's haematoxylin (3870, J.T.Baker® Chemicals - Avantor Performance Materials, Center Valley, USA) for 10 seconds and rinsed with demineralized water for 5 minutes. Subsequently, the slides were counterstained with filtered 0.4% Fast Green (F7252, Sigma-Aldrich, Saint Louis, USA) for 4 minutes, subjected to 1% acetic acid for two times 3 minutes, and stained with 0.125% aqueous Safranin O (58884, Sigma-Aldrich, Saint Louis, USA) for 7 minutes. The slides were then dehydrated through graded ethanol (70% and 96% both two times 1 minute; 100% ethanol two times 3 minutes) and xylene (two times 5 minutes) and mounted using Pertex mounting medium.

TOLUIDINE BLUE O STAINING

For the Toluidine Blue staining, the alginate bead sections were deparaffinized (see Safranin O/Fast Green staining) and subsequently washed for 1 minute in tap water. Thereafter the alginate bead slides were treated with 10 mM citrate buffer (pH 6) for 10 minutes to dissolve the alginate. Next the slides were subjected to 0.4% Toluidine Blue O (CI.52040, Sigma-Aldrich) for 3 minutes, after which they were rinsed in tap water for 1 minutes. After drying the slides they were dehydrated with graded ethanol (see Safranin O/Fast Green staining) and mounted using Pertex mounting medium.

COLLAGEN TYPE I & II IMMUNOHISTOCHEMISTRY

For collagen type II immunohistochemistry (IHC), the alginate bead sections were deparaffinized (see Safranin O/Fast Green staining). Subsequently the alginate bead slides were treated with 10 mM citrate buffer (pH 6) for 15 minutes to dissolve the alginate. Thereafter all slides were incubated with Dual Endogenous Enzyme Block (S2003, DAKO, Glostrup, Denmark) for 10 minutes to block endogenous peroxidase activity and washed two times for 5 minutes with PBS + 0.1% Tween (PBST 0.1%). Antigen retrieval was performed using 1 mg/mL pronase (11459643001, Roche Diagnostics, Almere, The Netherlands) and 10 mg/mL hyaluronidase (H3506, Sigma-Aldrich, Saint Louis, USA) in PBS for 30 minutes at 37 °C. Next, the slides were washed with PBST 0.1%. To reduce background interference the slides were blocked with 5% BSA in PBS for 30 minutes at RT. Thereafter, the samples were incubated overnight at 4 °C with collagen type II mouse monoclonal antibody (0.4 µg/mL; II-II6B3, DSHB, Iowa City, IA) in 5% BSA in PBS. For the negative control staining, the first antibody was replaced by normal mouse IgG1 (0.4 µg/mL; X 0931, Dako, Hamburg, Germany). The next day, the slides were washed with PBST0.1% before the slides were subjected to the secondary antibody (Brightvision Poly-HRP-Anti Mouse IgG, VWRKDPVM110HRP, Immunologic) for 60 minutes at RT. After washing with PBS, the slides were incubated with the Bright-DAB substrate kit (VWRKBSO4-116, Immunologic) for 5 minutes and counterstained with haematoxylin QS solution (H3404, Vector Laboratories, Burlingame, USA) for 1 min. Thereafter, they were washed with tap water for 10 minutes and dehydrated with graded ethanol (see Safranin O/Fast Green staining) and mounted using Pertex mounting medium. For collagen type I immunohistochemistry, the same procedure was followed with the collagen type I mouse monoclonal antibody (0.1 µg/mL; ab6308, Abcam, Cambridge, UK).

RESULTS

GENE EXPRESSION PROFILING

MONOLAYERS

The expression of NC marker *KRT8* was profoundly decreased in all culture conditions at day 14 compared with the NCs collected directly after digestion (day 0) (Fig. 4). When the monolayers were cultured in high glucose conditions, the expression of *KRT8* slightly decreased compared with the low glucose condition, and this effect was more pronounced when FCS was added (Fig. 4). Unexpectedly, the results show an opposite effect with regard to the expression of *KRT18,* which was increased in the NCs cultured in high glucose conditions and even more with the addition of FCS (Fig. 4). Yet another effect was observed with regard to the expression of *KRT19,* which was profoundly decreased after 14 days of culture as well compared with day 0, but was higher in the NCs cultured in high glucose conditions compared with the low glucose conditions and the addition of FCS (Fig. 4). *CA* expression was also increased in the NCs cultured in high glucose conditions compared with the low glucose conditions and was the lowest in the cells cultured with FCS (Fig. 4). The same trend was demonstrated with regard to the expression of apoptotic marker *CASP3*. *ACAN* expression was decreased when the cells were cultured for 14 days compared with the day 0 samples (Fig. 4). Furthermore, the expression of *ACAN* was decreased in the samples cultured in high glucose levels compared with the expression of the samples in the low glucose condition and decreased even more when FCS was added (Fig. 4). The expression of the other genes mentioned in Table 2 were not detectable.

ALGINATE BEADS

The expression of NC marker *KRT8* was profoundly decreased in all culture conditions at day 14 compared with day 0 (Fig. 5). When the alginate beads were cultured in high glucose, the expression of *KRT8* was slightly increased compared with the beads cultured in low glucose concentration, whereas the addition of FCS resulted in a slight decrease (Fig. 5). The expression of NC maker *KRT18* was increased in the alginate beads cultured for 14 days in low and high glucose conditions compared with the samples collected at day 0 (Fig. 5). There was a slight decrease in *KRT18* expression when the glucose concentration was increased and a pronounced decrease when FCS was added (Fig. 5). After 14 days of culture, the alginate beads in all culture conditions expressed almost no *KRT19* anymore (Fig. 5). The expression of *CA* was increased in the alginate beads cultured in high glucose compared with the NCs collected at day 0, whereas the expression was decreased in de NCs cultured in low glucose and almost negligible in the NCs cultured in high glucose + FCS (Fig. 5). Gene expression of *ACAN* was decreased after 14 days of culture in low glucose conditions and even more in high glucose conditions, whereas with the addition of FCS the expression resulted in the least pronounced decrease (Fig. 5). The expression of the other genes mentioned in Table 2 were not detectable.

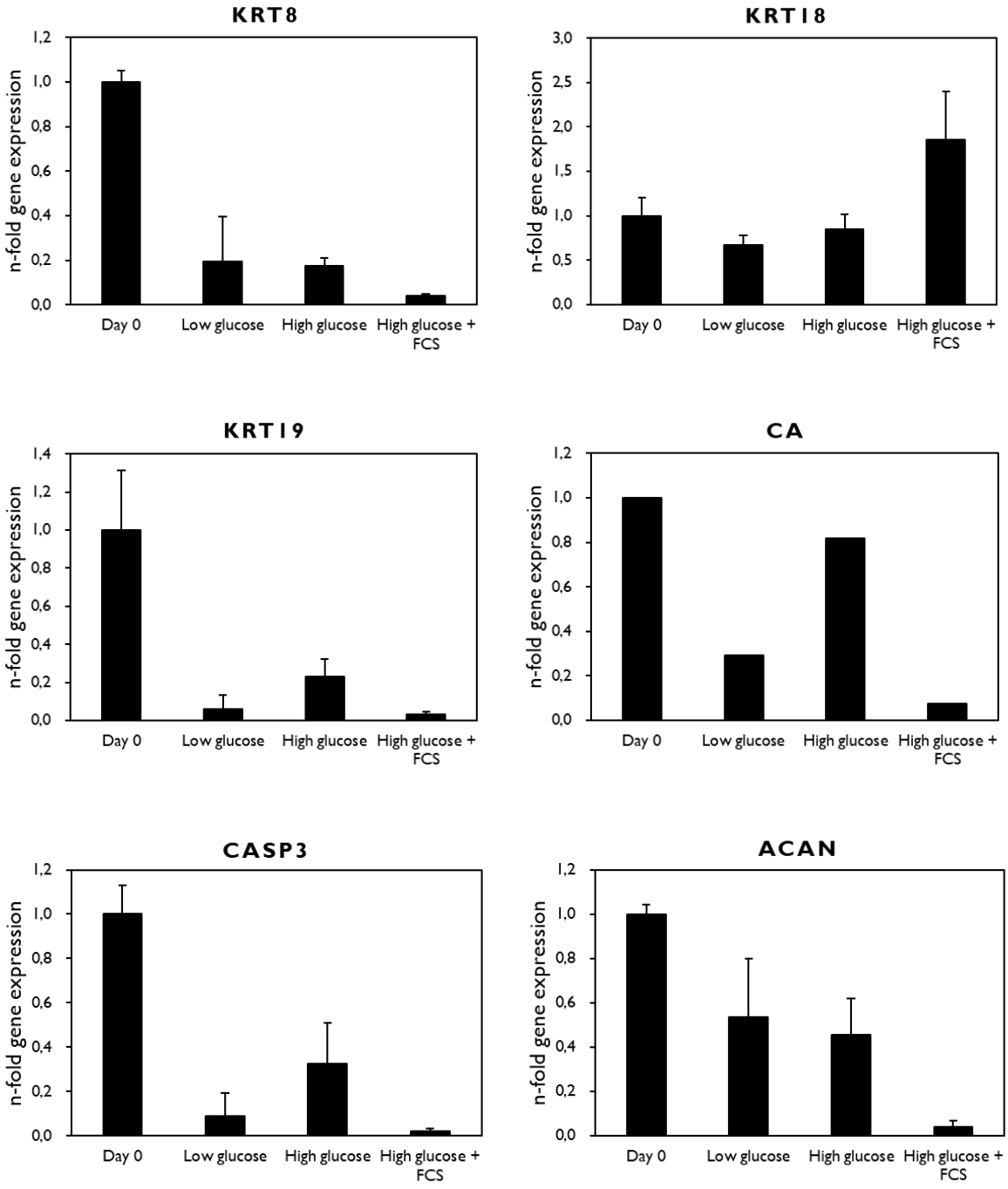

Figure 4: No distinct adverse effect of the high glucose conditions both with and without FCS was encountered with regard to the expression of different NC markers, apoptotic, and extra-cellular matrix associated genes compared to the low glucose conditions. Relative *KRT8, KRT18, KRT19, CA, CASP3,* and *ACAN* gene expression (mean + SD) of the **monolayers** derived from porcine NCs cultured for 14 day in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)). The samples collected directly after tissue digestion (day 0 of culture) were set at 1. n = 1; biological triplicates per condition.

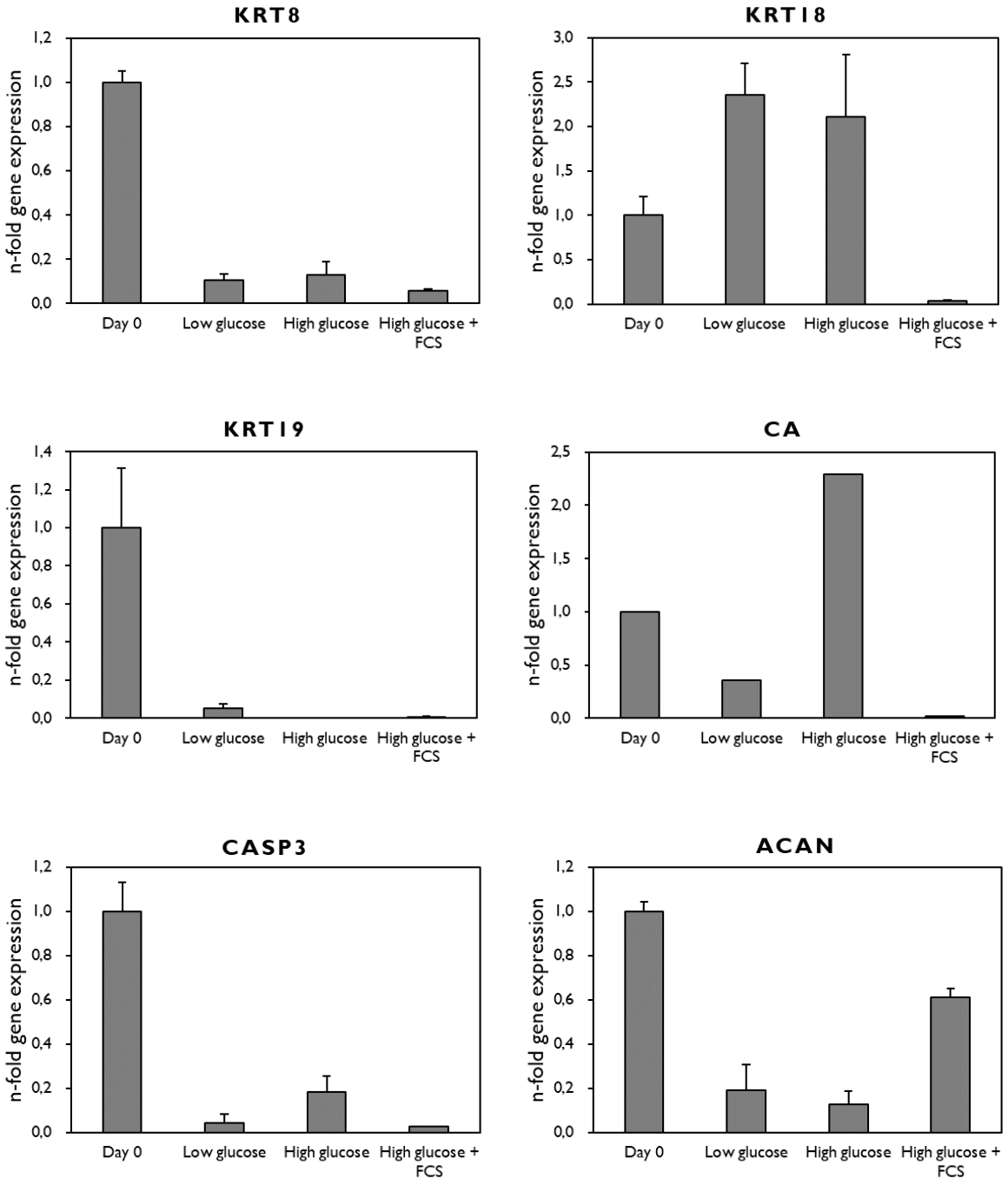

Figure 5: No distinct adverse effect of the high glucose conditions both with and without FCS was encountered with regard to the expression of different NC markers, apoptotic, and extra-cellular matrix associated genes compared to the low glucose conditions. Relative *KRT8, KRT18, KRT19, CA, CASP3,* and *ACAN* gene expression (mean + SD) of the **alginate beads** derived from porcine NCs cultured for 14 day in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)). The samples collected directly after tissue digestion (day 0 of culture) were set at 1. n = 1; biological triplicates per condition.

GAG & DNA MEASUREMENTS

MONOLAYERS

The DNA content of the monolayers cultured in high glucose medium was slightly decreased compared with the monolayers cultured in low glucose conditions and even lower when FCS was added (Fig. 6A). The GAG content of the monolayers cultured with FCS was lower than that of the monolayers cultured in low glucose, whereas the GAG content of the monolayers cultured in serum-free high glucose medium was the lowest (Fig. 6B). The NCs cultured with FCS produced more GAGs when corrected for the DNA content compared with the NCs in low glucose conditions, whereas the GAG production per DNA was decreased when the NCs where cultured in serum-free high glucose medium (Fig. 6C). The GAG release in the medium in the second week of the culture was the highest of the monolayers cultured in high glucose conditions (Fig. 6D). The total GAG production was slightly increased in the monolayers cultured in high glucose conditions with FCS compared with both the serum-free conditions (Fig. 6E). The overall results of the total GAG production show more release than incorporation of GAGs in the monolayers (Fig. 6E).

ALGINATE BEADS

The DNA and GAG content of the monolayers cultured in high glucose concentration were increased compared with the low glucose condition, whereas these were slightly decreased when FCS was added (Fig. 7A,B). The GAG production corrected for the DNA content was slightly decreased in the alginate beads cultured in high glucose conditions compared with the production of the alginate beads in low glucose, whereas addition of FCS resulted in a slight increase (Fig. 7C). The GAG release in the medium in the second week of the culture was increased in the high glucose condition and even more with the addition of FCS (Fig. 7D). The total GAG production of the alginate beads cultured in the high glucose environment was increased compared with the production of the alginate beads cultured in low glucose conditions and with FCS (Fig. 7E). The overall results of the total GAG production show more incorporation of GAGs in the alginate beads compared with the release (Fig. 7E).

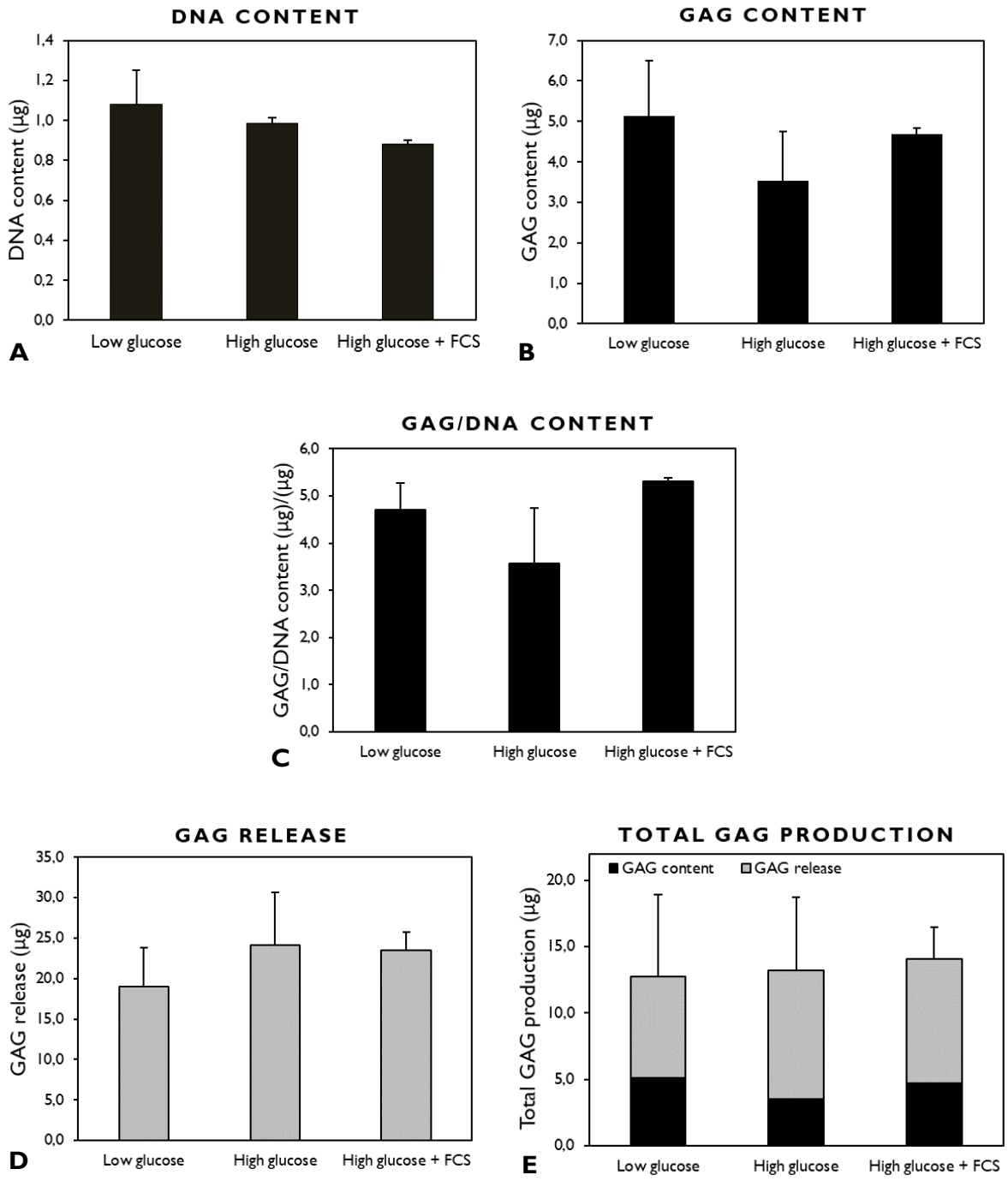

Figure 6: The DNA and GAG content were slightly decreased in the high glucose conditions and with the addition of FCS compared with the low glucose condition. GAG and DNA content (mean + SD) of the **monolayers** derived of porcine NCs cultured for 14 day in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)). (a) DNA content of the monolayers. (b) GAG content of the monolayers. (c) GAG content corrected for the DNA content. (d) Total amount of GAGs released in the culture medium by the monolayers from day 7. (e) Total GAG production (GAG content monolayers and amount of GAGs released taken together). n = 1; biological triplicates per condition.

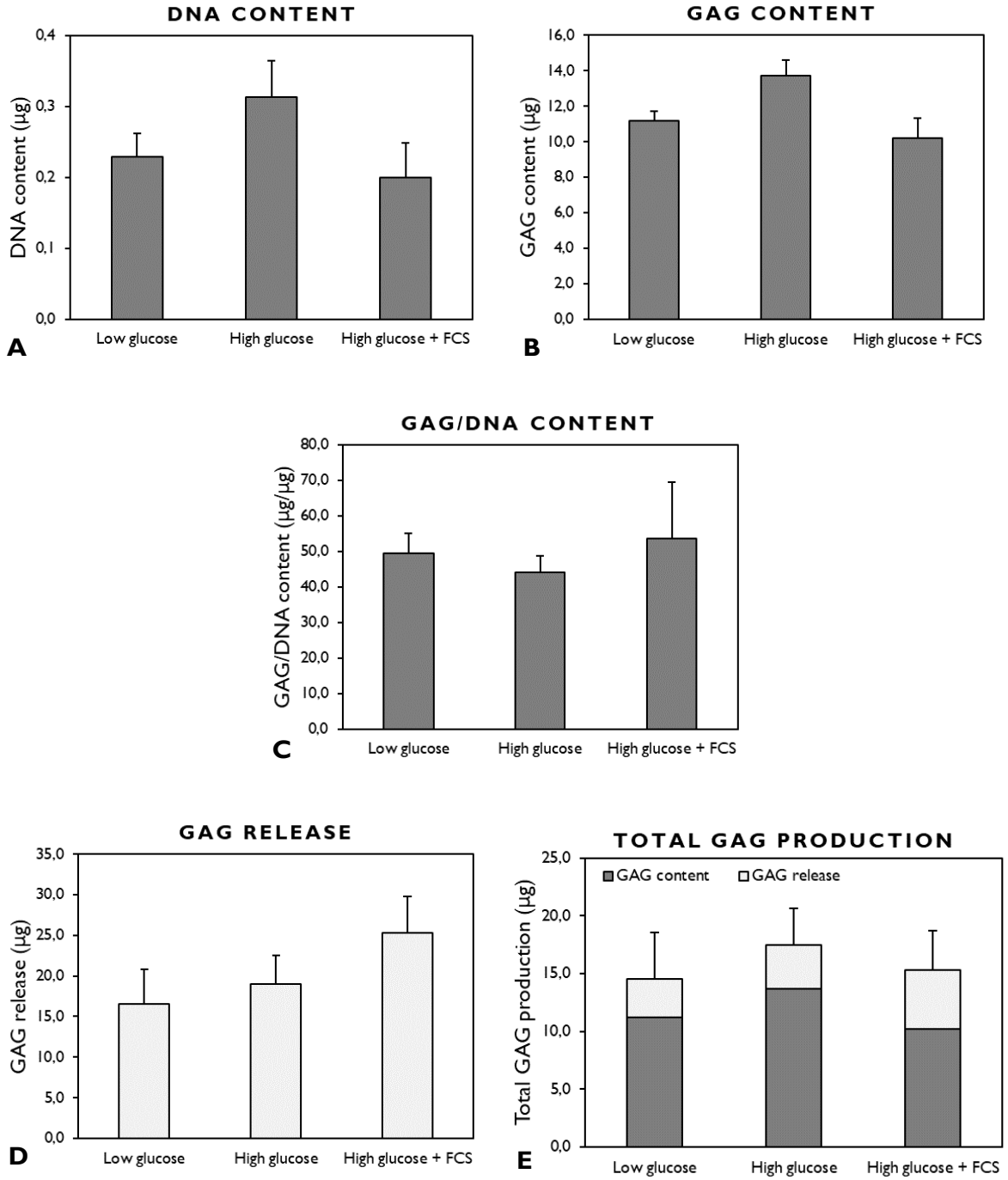

Figure 7: The DNA and GAG content were increased in the high glucose conditions whereas these were slightly decreased by the addition of FCS compared with the low glucose condition. GAG and DNA content (mean + SD) of the **alginate beads** derived of porcine NCs cultured for 14 day in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)). (a) DNA content of the alginate beads. (b) GAG content of the alginate beads. (c) GAG content corrected for the DNA content. (d) Total amount of GAGs released in the culture medium by the alginate beads from day 7. (e) Total GAG production (GAG content alginate beads and amount of GAGs released taken together). n = 1; biological triplicates per condition.

HISTOLOGY

CELL MORPHOLOGY

Morphological examination directly after NP tissue digestion showed that the cell population primarily consisted of round large cells which were mostly organized in large clusters and contained vacuoles, that is, characterized as NCs (Fig 8A). Evaluation of the histology of the alginate beads cultured in the serum-free conditions on day 14 showed that the majority of the cell population consisted of clusters of cells with a relatively large amount of vacuolated cytoplasm, that is, an NC-like phenotype (Fig. 8B). Unfortunately, the evaluation of cell morphology within the monolayers cultured without FCS was not accurately possible because the clusters contained multiple layers of cells obscuring their morphological characteristics (Fig. 8B). The cells cultured with FCS showed predominantly a spindle-shaped phenotype and a loss of vacuoles both in the monolayer as well as in the alginate beads (Fig. 8B).

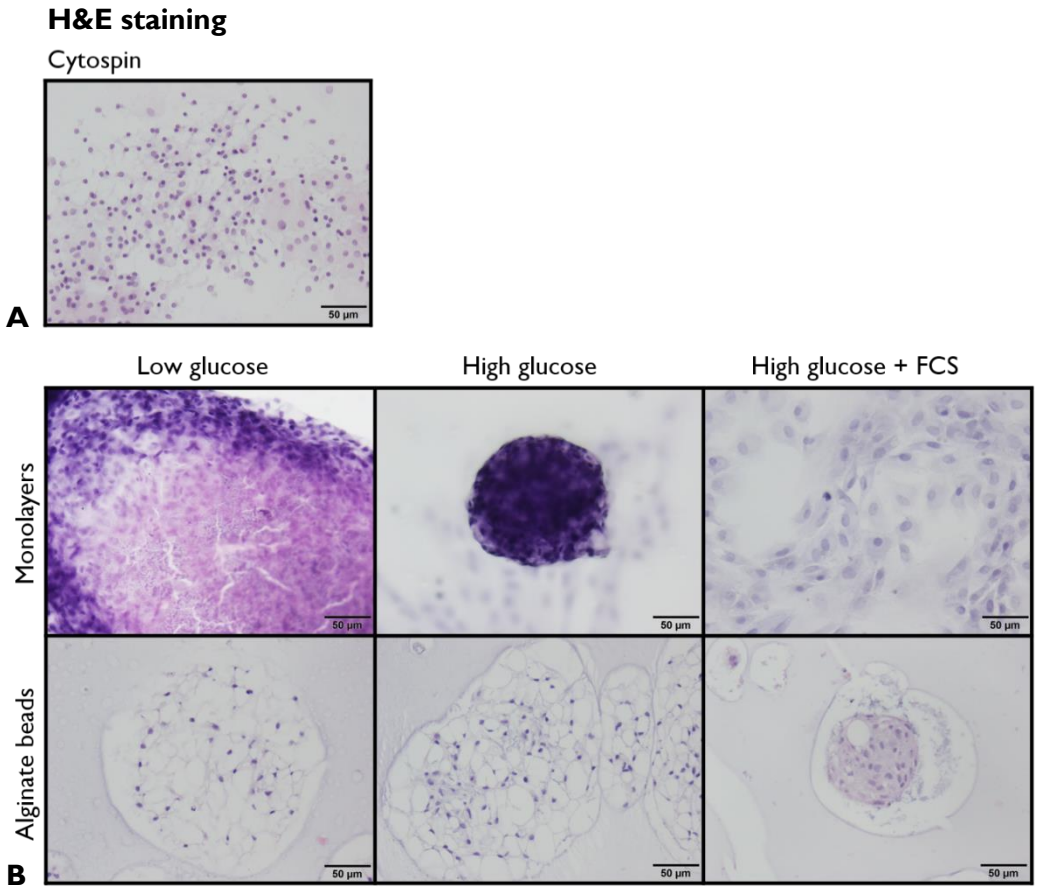

Figure 8: Representative histology images of (a) a cytospin slide collected directly after digestion (day 0) and (b) monolayers (upper row) and alginate beads (bottom row) cultured for 14 days in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)) stained with hematoxylin and eosin (H&E). Morphological examination of the cytospin showed mainly NCs which were organized in large clusters. Evaluation of the histology on day 14 showed that the cell population of alginate beads cultured in low and high glucose conditions consisted of clusters of cells with a NC specific phenotype. The cells cultured with FCS showed predominantly a spindle-shaped phenotype and a loss of vacuoles both in the monolayers as well as in the alginate beads.

EXTRACELLULAR MATRIX DEPOSITION

In contrast with the DMMB assay, the Safranin O/Fast Green staining revealed only slight deposition of GAGs in the monolayers cultured in low and high glucose and an absence of GAGs when FCS was added (Fig. 9). The Toluidine Blue O staining demonstrated most extracellular matrix deposition in the low glucose condition and with the presence of FCS, which is more in line with the results of the DMMB assay (Fig. 10). The Safranin O/Fast

Green staining and Toluidine Blue O staining of the alginate beads showed no clear differences in GAG production between the different conditions (Fig. 9,10). Immunohistochemistry of the monolayers showed slight collagen type I protein deposition in all culture conditions (Fig. 11) and a pronounced deposition of collagen type II protein, particularly in the monolayers cultured in the serum-free media (Fig. 12). Immunohistochemistry of the alginate beads revealed no deposition of collagen type I protein (Fig. 11) and only a slight deposition of collagen type II protein in all culture conditions (Fig. 12).

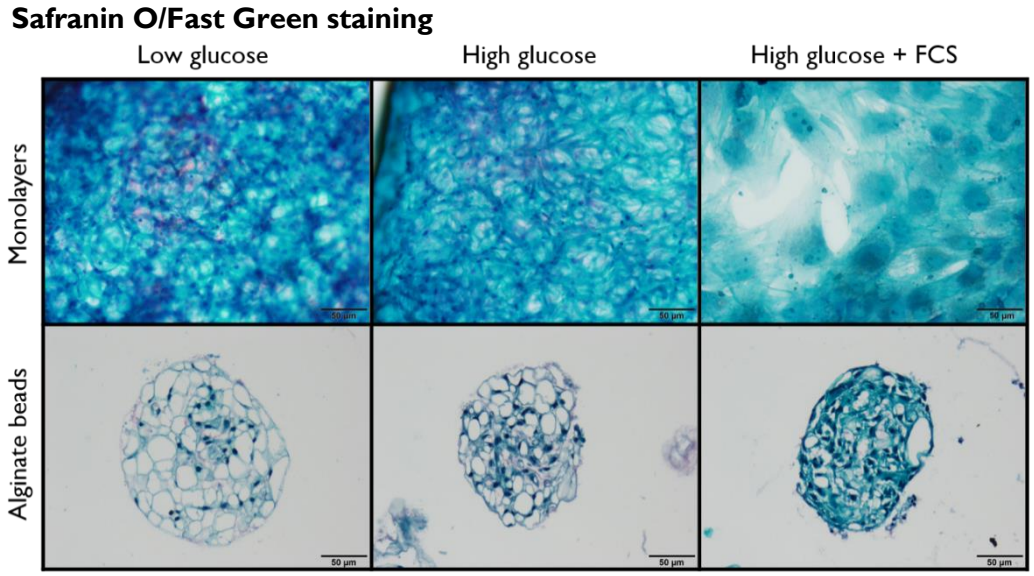

Figure 9: Representative histology images of the monolayers (upper row) and alginate beads (bottom row) cultured for 14 days in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)) stained with Safranin O/Fast Green. The staining revealed only a slight deposition of GAGs in the monolayers cultured in low and high glucose and the absence when FCS was added. There was no clear difference in the GAG productions of the alginate beads in the different conditions.

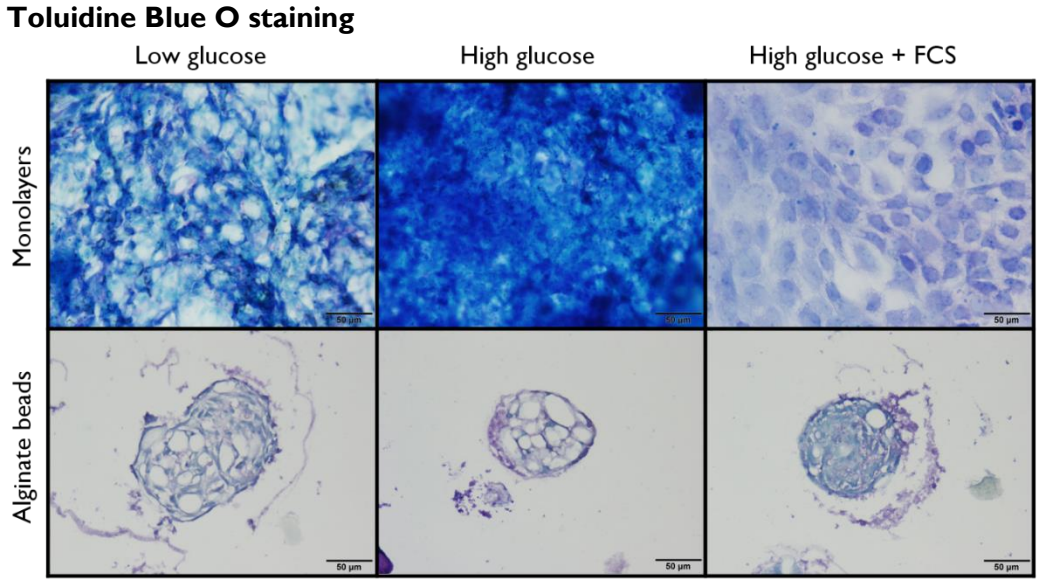

Figure 10: Representative histology images of the monolayers (upper row) and alginate beads (bottom row) cultured for 14 days in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)) stained with Toluidine Blue O. The staining demonstrated the most extracellular matrix deposition in the low glucose condition and with the presence of FCS in the monolayers. There was no clear difference in the GAG productions of the alginate beads in the different conditions.

Collagen type I IHC

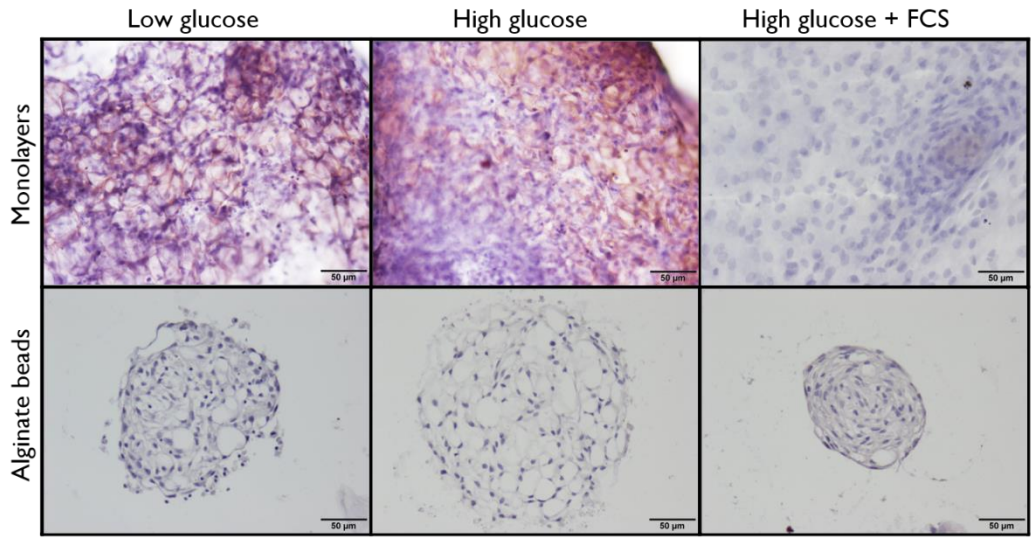

Figure 11: Representative histology images of the monolayers (upper row) and alginate beads (bottom row) cultured for 14 days in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% fetal calf serum (FCS)) stained with immunohistochemical staining for collagen type I protein. Immunohistochemistry of the monolayers showed slight collagen type I protein deposition in all culture conditions, whereas the alginate beads demonstrated no deposition of collagen type I protein.

Collagen type II IHC

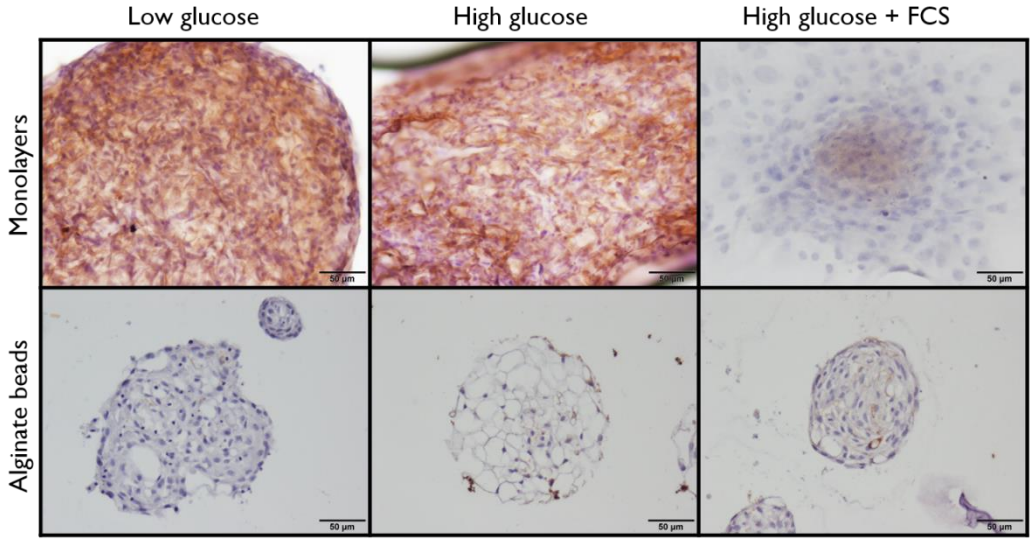

Figure 12: Representative histology images of the monolayers (upper row) and alginate beads (bottom row) cultured for 14 days in different culture conditions (Low glucose: α-MEM; High glucose: α-MEM + 0.1 M D-glucose; High glucose + FCS: α-MEM + 0.1 M D-glucose + 10% FCS) stained with immunohistochemical staining for collagen type II protein. Immunohistochemistry of the monolayers showed a pronounced deposition of collagen type II particularly when cultured in serum-free medium. Immunohistochemistry of the alginate beads revealed only a slight deposition of collagen type II protein in all culture conditions.

DISCUSSION

This study aimed at generating and validating a culture system in which porcine NCs lose their specific phenotype in a controlled manner. To accomplish this, different culture conditions were used based upon results of previously conducted studies with regard to the effect of different culture conditions on the perseverance of the NC specific phenotype.[18,19,23-27] The experimental groups of the current study included low glucose conditions (α-MEM: 0.006 M D-glucose), high glucose conditions (α-MEM + 0.1 M D-glucose) and the additional effect of FCS (α-MEM + 0.1 M D-glucose + 10% FCS) in both a 2D (monolayer) as 3D (alginate bead) culture system.

EFFECTS OF INCREASING THE GLUCOSE CONCENTRATION

The results of the present study revealed no pronounced effect of increasing the glucose concentration on the NC specific morphology. Furthermore, no clear effects were discovered with regard to the expression of phenotype-related genes by culturing the NCs in 0.1 M glucose conditions. Only the expression of *ACAN* was slightly decreased in both the monolayers and alginate beads cultured in a high glucose environment, indicating less production of healthy matrix, which was confirmed by the results of the DMMB assay of the monolayers. However, the results of the DMMB assay of the alginate beads, Safranin O/Fast Green, and Toluidine Blue O staining were not in line with the aforementioned suggestion. These findings are in contrast with the results of the study of Spillekom *et al.* (2014), in which several differences were observed between NCs cultured in standard DMEM/F12 (31331; Invitrogen) and NCs cultured in a-MEM (22561; Invitrogen). In their study, differences in expression of several genes indicated that NCs cultured in alginate beads in a-MEM retained their phenotype better than NCs cultured in DMEM/F12.[19] It was hypothesized that this improved perseverance of phenotype in a-MEM was mainly due to the lower concentration of D-glucose than in DMEM/F12 (5.5 vs. 17.5 mM, respectively). The authors suggested that since IVD cells are subject to low glucose concentrations *in vivo*,[20] the relatively low glucose content in a-MEM may facilitate NCs to retain their phenotype.[19] An explanation for the discrepancy between their results and the results of the current study could be the fact that in the study of Spillekom *et al.* (2014) the alginate beads were cultured in DMEM/F12 with an osmolarity of 300 mOsm/L instead of 400 mOsm/L used in the present study. Furthermore, in their study 10% FCS was used in all the culture conditions. So it could be that the loss of phenotype was a combination of all these factors together instead of only the increased concentration of glucose in DMEM/F12. Additionally, except for the glucose concentration, a-MEM and DMEM/F12 also differ regarding the presence of ascorbic acid and the concentration of other vitamins (*e.g.*, biotin), various inorganic salts, and amino acids, which could also have influenced the functionality of NCs in culture. Altogether, the present study provides a more reliable indication of the effect of increasing the glucose concentration, since this was the only variable in the two experimental groups in contrast to the use of two different kinds of culture medium. Another explanation for the discrepancy between the effect of high glucose in the current study and that of Spillekom *et al.* (2014) is the difference in species used, since in the current study porcine NCs were used instead of canine NCs.

Hyperglycemia has been positively correlated with IVD degeneration in diabetic rats[21] and it has been shown that a high glucose environment stimulates apoptosis and inhibits proliferation of NCs *in vitro*.[22] The decreased DNA and GAG content and the increased expression of apoptotic marker *CASP3* of the monolayers cultured in high glucose conditions in the current study are in line with the aforementioned studies. However, the alginate beads showed no decrease in DNA and GAG content, but show a slight increase in expression of *CASP3* when cultured in high glucose conditions as well. An explanation for the less pronounced effect of the high glucose conditions in the alginate beads could be that they are less susceptible for changes in the culture conditions, because different studies show that NCs thrive better in 3D culture systems.[23,25]

Taken together, the present study demonstrates no profound effect of increasing the glucose concentration from 0.006 to 0.1 M on the de-differentiation of NCs in culture. Although aforementioned studies provide evidence that NCs may thrive best in a low glucose environment, it is also suggested that NCs demand more energy and are less resistant to nutritional deprivation than CLCs[30] which could explain why increasing the glucose level did not result in a pronounced loss of phenotype of cultured NCs.

EFFECTS OF THE ADDITION OF FCS

A striking observation in the current study was that the monolayers were only adhering to the uncoated culture surfaces when FCS was added to the culture medium. It could be that the NCs were not able to adhere to the surfaces because of their cluster formation rich in ECM and that due to the addition of FCS the subsequent loss of cluster formation and ECM enabled the cells to adhere better to the culture surface. This hypothesis is confirmed by the drop in *ACAN* expression of the monolayers cultured with FCS, although the GAG content measured with the DMMB assay was not decreased. A possible explanation for this discrepancy could be that the GAG content was measured at day 14 and because of the transfer of the serum-free experimental groups to coated surfaces, the cells in all groups had already adhered at that time-point. Additionally, it could be that with this transfer at day 7 GAGs were lost. This is supported by the observation that the GAG content of the alginate beads cultured with FCS showed a decreased GAG content and increased GAG release, which could indicate an increased catabolism of healthy ECM. Additionally the study of Arkesteijn *et al*. (2016) also showed a decrease of *ACAN* expression when NCs were cultured with FCS.[27] However, to confirm this suggestion other ECM components present in the NC clusters (*e.g.* laminin) should be taking into account as well. Furthermore, since it is suggested that FCS contains adhesion-promoting properties due the presence of cell attachment-promoting proteins *e.g.* fibronectin and vitronectin[31] this could also be a reason for the lack of adherence of the NCs cultured without FCS in the present study.

The DNA content of both the monolayers and alginate beads cultured with FCS was slightly decreased. This is in line with the study of Arkesteijn *et al.* (2016) in which decreased cell viability was observed by culturing with FCS.[27] Also, the NC phenotype was better retained in serum-free media.[27] In the present study, we show that the NCs cultured in serum-free medium preserved their cluster formation and their vacuolated cytoplasm, whereas when FCS was added to the medium, a more spindle-shaped phenotype developed in both the monolayers and alginate beads after 14 days of culture as well. As FCS contains a multitude of undefined factors[32], it appears that these can have a deteriorating effect on NC morphology and phenotype. The study of Rastogi *et al.* (2009) demonstrates that NCs retained their phenotype better when cultured with only 2% instead of 10% FCS[33], which supports the results of the current study and the observations of Arkesteijn *et al.* (2016).[27] However, the study of Gantebein *et al.* (2014) reported a trend towards an increased GAG/DNA ratio when NCs were kept in medium containing 10% FCS compared to a serum-free medium.[26] This discrepancy could be due to the fact that they used Dulbecco's High Glucose Modified Eagle Medium (HG-DMEM) instead of α-MEM. α-MEM is better able to retain the NC phenotype than DMEM, which could be explained by the presence of ascorbic acid in α-MEM.[33] Ascorbic acid is also naturally present in FCS.[34] Possibly, the cells cultured in HG-DMEM alone performed worse because of the lack of ascorbic acid and not necessarily because of the other components of FCS.

Although the use of FCS to de-differentiate NCs seems promising, it should be taken into account that FCS is an ill-defined cocktail[34] and cells may respond differently to each batch, decreasing the reproducibility.[27] The study of Arkesteijn *et al.* (2016) showed that stripped FCS, which is relatively poor in growth factors due to charcoal treatment, had less negative effects on NCs, suggesting that unidentified growth factors in FCS are responsible for its effect on the NC morphology.[27] Therefore, it would be interesting to unravel which exact factors are contributing to this effect so that they can be used separately to create a culture system in which the NCs lose their specific phenotype in a more controlled manner.

EFFECTS OF THE CULTURE SYSTEM

In the current study, both a 2D (monolayer) as well as 3D (alginate bead) culture system was used. Although several previously conducted studies show that NCs preserve their specific morphology better in a 3D culture system[23,25], the results of the present study show quite similar responses of the NCs in both culture systems. The porcine NCs were able to retain their cluster formation and NC morphology in both the serum-free media (low and high glucose) cultured in monolayers as well as in alginate beads. Additionally, as stated above, the effect of FCS on the NC morphology was similar in both culture systems resulting in more spindle-shaped cells and a loss of vacuoles. An explanation for the discrepancy between the results of the present study and the studies of

Erwin *et al.* (2009)[23] and Smolders *et al.* (2012)[25] could be that in their studies DMEM/F12 and FCS were used which is possibly the main reason for the loss of the NC specific morphology instead of the 2D culture system.[23,25]

However, there were some dissimilarities in the results of the GAG and DNA measurements between the two culture systems. The GAG and DNA content of the NC alginate beads cultured in high glucose were increased whereas those of the monolayers were decreased compared to the NCs cultured in low glucose conditions. As mentioned before, an explanation for this discrepancy in response to the glucose conditions could be that in alginate beads the NCs are less susceptible for changes in the culture conditions because it is shown that they thrive better in 3D culture systems.[23,25] Since it is also reported that NCs demand more energy and are less resistant to nutritional deprivation than CLCs[30] it could be that the high glucose level has actually a beneficial effect on the NCs in the alginate beads. There was also a discrepancy in the effect of FCS on the GAG content of the NCs cultured in the 2D system compared to the 3D culture system, since in the alginate beads a drop in GAGs was encountered in contrast to the monolayers. This could be due to the fact that the NC monolayers cultured in the serum-free conditions were transferred at day 7 and it could be that GAGs were lost during this process. Lastly, the present study shows that with regard to the total GAG production of the alginate beads the GAG incorporation contributed to a bigger extent than the release whereas this ratio was reversed in the monolayers. An explanation for this could be that the NCs are better capable of incorporating GAGs in a 3D system than in an 2D system, while the release of GAGs could be hampered by the alginate.

LIMITATIONS & FUTURE PERSPECTIVES

Since the present experiment was a pilot study during which various hurdles were encountered, quite some limitations and suggestions for future studies should be discussed. First of all, this experiment included only 3 biological replicates of a pooled sample of NCs of two different pig spines. Therefore the small sample size used in this study hampered profound (statistical) analyses of the obtained data, so future studies should include samples of more donors.

Another limitation was the inaccurate counting of the cells, since the automatic cell counter (TC20™, Bio-Rad) is not able to count cells in clusters. For this reason, NC clusters in follow up studies should be counted with the aid of a NucleoCounter®, which is able to count the nuclei before and after lyses of the cells and in that manner can determine the amount of live and death cells before starting the culture.

Another hurdle which was encountered during this study was the adherence of the NC monolayers to the culture surfaces. Up until day 7 of the culture, the NC clusters without FCS did not adhere to the uncoated surfaces of the 48 wells plate and the chamber slides. The study of Smolders *et al.* (2011) demonstrated that canine NCs adhered to the culture surfaces at day 4, however, in this study all NCs were subjected to 10% FCS.[25] To overcome this problem in the present study, the well surfaces were coated with 20 µg/mL laminin-521[24] at day 7 whereafter the NC clusters were transferred and consequently adhered firmly to the 48 wells plate but still had difficulties with finding grip on the glass 8-well chamber slides. This was presumably due to the more smooth surface and different substrate stiffness.[24] It cannot be excluded that this transfer of the clusters halfway through the culture period had an influence on the behavior of the NCs. Furthermore, since the clusters cultured with FCS were not exposed to laminin, it should be taken into account that there was an extra variable with regard to the different experimental groups which hampers reliable analyses. To prevent these variations, future culture set-ups should include laminin coating in each experimental group from the start of the experiment and the use of plastic chamber slides should be considered or coating of glass chamber slides with another substrate *e.g.* fibronectin or gelatin.[24]

Unfortunately, the evaluation of cell morphology and the (immunohistochemical) stainings within the monolayers cultured without FCS was not accurately possible because the clusters contained multiple layers of cells obscuring their morphological characteristics. Furthermore, it is possible that during IHC of these 3D structures, the Bright-DAB substrate was accumulated in the clusters which could have caused false positive results. To overcome this problem, immunofluorescence confocal microscopy could be used in follow up studies to obtain an overview of the morphology and presence of different proteins within these 3D NC clusters.

The expression of several tested genes in the present study were undetectable, while others did not always match with the other read-out parameters. A possible explanation for these unexpected results could be the low input of cDNA, another explanation could be that gene expression was determined at a time point of low expression. So future studies should take more time points into account to determine the optimal timing for gene expression profiling. Furthermore, ongoing work focusses on optimizing primers for gene expression profiling of more different genes (*e.g. brachyury, notochord homeobox*, and *collagen type I and II*) and antibodies accounting for more markers using immunohistochemistry/immunofluorescence (*e.g.* cytokeratin-8, -18, -19, brachyury, and notochord homeobox).

As mentioned before, the use of FCS to de-differentiate NCs seems promising, but it should be taken into account that FCS is an ill-defined cocktail[34] and cells may respond differently to each batch, decreasing the reproducibility.[27] Since the study of Arkesteijn *et al.* (2016) provides evidence that unidentified growth factors in FCS are responsible for its effect on the NC morphology[27], future studies should focus on confirming this using stripped FCS and/or delineating the effect of specific growth factors *e.g.* TGF-β_1.

CONCLUSIONS

In conclusion, the results of the current study suggest that FCS induces de-differentiation of NCs in both a 2D and 3D culture system, whereas increasing the glucose concentration did not affect NC morphology. However, since the composition of FCS is uncertain and batches can vary, future research should delineate which specific growth factor(s) can be used to induce a more controlled de-differentiation. Furthermore, it should be mentioned that because of the limitations of this pilot study, it is yet infeasible to draw definite conclusions with regard to the optimal de-differentiation culture set-up. Therefore, follow up studies should delineate whether the discussed improvements will result in a valid culture system to provide for the material to perform (epi)genetic analysis. When succeeded, this will hopefully contribute to the optimize the differentiation of iPSCs to NLCs.

APPENDIX

Attended Congresses:

- 'Mini Symposium Bone Regenerative Medicine'
- 'European Veterinary Conference Voorjaarsdagen'
- 'Symposium Expanding the Regenerative Medicine Utrecht Community'
- '12th Dutch Stem Cell Meeting'

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