RESEARCH ARTICLE

Recommendations for intervertebral disc notochordal cell investigation: From isolation to characterization

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Abstract

Background: Lineage-tracing experiments have established that the central region of the mature intervertebral disc, the nucleus pulposus (NP), develops from the embryonic structure called "the notochord". However, changes in the cells derived from the notochord which form the NP (i.e., notochordal cells [NCs]), in terms of their phenotype and functional identity from early developmental stages to skeletal maturation are less understood. These key issues require further investigation to better comprehend the role of NCs in homeostasis and degeneration as well as their potential for regeneration. Progress in utilizing NCs is currently hampered due to poor consistency and lack of consensus methodology for in vitro NC extraction, manipulation, and characterization.

Methods: Here, an international group has come together to provide key recommendations and methodologies for NC isolation within key species, numeration, in vitro manipulation and culture, and characterization.

Results: Recommeded protocols are provided for isolation and culture of NCs. Experimental testing provided recommended methodology for numeration of NCs. The

issues of cryopreservation are demonstrated, and a pannel of immunohistochemical markers are provided to inform NC characterization.

Conclusions: Together we hope this article provides a road map for in vitro studies of NCs to support advances in research into NC physiology and their potential in regenerative therapies.

KEYWORDS

culture systems, development, intervertebral disc, notochordal cells, nucleus pulposus, tissue-specific progenitor cells

1 | INTRODUCTION

The notochord is a mesodermal midline structure (also called the axial mesoderm) located along the anterior–posterior axis at the ventral surface of the neural tube and dorsal to the gut, characteristic of chordates.[1,2] It is a transient signaling structure involved in the regionalization and fate of the surrounding embryonic tissues.[3,4] In vertebrates, the notochord plays a key role in signaling and coordinating the development of the vertebral column.[5,6] During development, the notochordal plate folds to develop into the neural tube forming the notochord (ventrally), which is flanked by the paraxial mesoderm and ectoderm (dorsally).[7,8] The paraxial mesoderm goes on to form the somites, which further differentiate into skeletal muscle, connective tissue, dermis, and the sclerotome. The latter eventually gives rise to the ribs, vertebral bodies, cartilaginous end plates (CEPs), and the annulus fibrosus (AF) of the intervertebral disc (IVD).[8–11] Whilst lineage tracing studies in mouse models have demonstrated that the founder cells of the nucleus pulposus (NP), the core of the IVD, originate in the embryonic notochord.[12,13] Once the notochord regresses, notochordal cells (NCs) are restricted to within the NP, and excluded from the forming vertebral bodies.[14]

Substantial changes in the cytomorphology and function of NCs are observed throughout morphogenesis, growth, and maturation of the IVD, from its initial state as a notochord primordium to its mature state: the NP of the adult disc. A consensus terminology is currently lacking for defining the notochordal-derived cells within the NP from development to adulthood. Here, we adopt the terminology utilized in Bach et al.,[15] where NCs from the embryonic notochord are defined as embryonic NCs (eNCs), whilst those within the central region of the IVD are termed notochordal cells (NCs).

As well described, at an early stage, the embryonic notochord is composed of large eNCs packed within the perinotochordal sheath consisting of extracellular matrix (ECM) proteins (mainly laminin, proteoglycans, and collagen). eNCs synthesize and secrete collagens and proteoglycans (containing chondroitin 4-sulfate, chondroitin 6-sulfate, and heparan sulfate GAG) which accumulate in the perinotochordal sheath.[16–18] Once these eNCs mature within the NP they exhibit multiple large cytoplasmic vacuoles and eventually differentiate into the NCs. With further NP maturation a transition in cell phenotype takes place from large vacuolated NCs, present in clusters, to smaller and more dispersed nonvacuolated NP cells (NPCs), although the timing of

this differs considerably by species (Figure 1).[19–37] The NPCs are phenotypically characterized by their ability to synthesize the appropriate matrisome[38] and can to some extent be identified by specific gene biomarkers.[29,39–41] Although differential gene markers are difficult to identify between the vacuolated NCs and mature nonvacuolated NPCs, the advent of single-cell transcriptomic analysis of these cells provides new perspectives and potential markers.[19,42–45] Thus, further research into developmental studies and NC biology is essential to provide insights into NC-related pathogenesis of IVD degeneration and to understand their potential in IVD regeneration.[8,20,46–49]

The NC population in the NP undergoes species-specific changes (Figure 1). In humans, NCs start to decline before birth (Figures 1 and 2), with complete loss based on their vacuolated morphology by teenage years. Richardson et al.,[19] reported the presence of NC remnants within adolescent human IVDs. Similarly, mature NPs from sheep, goats, and cows contain no NCs, whereas in mice, rabbits, rats, and pigs, the NC population is maintained until much later in life (Figures 1 and 3).[29,50] In dogs, NCs are lost at about 1 year of age in chondrodystrophic breeds (CD; e.g., Beagles and Dachshunds), but remain in the NP until middle/old age in nonchondrodystrophic (NCD) breeds (e.g., Shepherds and Mongrels; Figures 1 and 3).[29,51,52]

The loss of the NC population in certain species (e.g., CD dogs) coincides with the development of IVD degenerative changes and clinical disease,[53] indicating that NCs may play a role in maintaining healthy NP tissue. Several studies[22,54,55] have determined the regenerative potential of NCs and found that they may be a promising target for regenerative and/or symptom-modifying therapies for IVD disease which has been reviewed recently.[15] However, <3% of papers reporting the potential use of cells to promote regeneration of the disc, utilized NCs as the cell choice,[56] whereas a larger emphasis has been on other cell types such as bone marrow-derived mesenchymal stem cells, and mature NP or AF cells. Studies investigating the use of NCs have sourced these from pigs,[55,57–59] rats,[60–62] rabbits,[60] and dogs.[63] The studies mainly utilized 3D in vitro culture, where the phenotype could be maintained at least partially, with a distinct lack of progression into clinical studies. Their limited use in research so far has been due to the difficulties in their utilization in vitro. In particular, maintaining phenotype in monolayer culture in vitro is problematic[55,58,64] and due to their limited capacity to proliferate without loss of phenotype, it is often difficult to harvest sufficient numbers for in vitro manipulation[64–66] and extraction itself often leads to
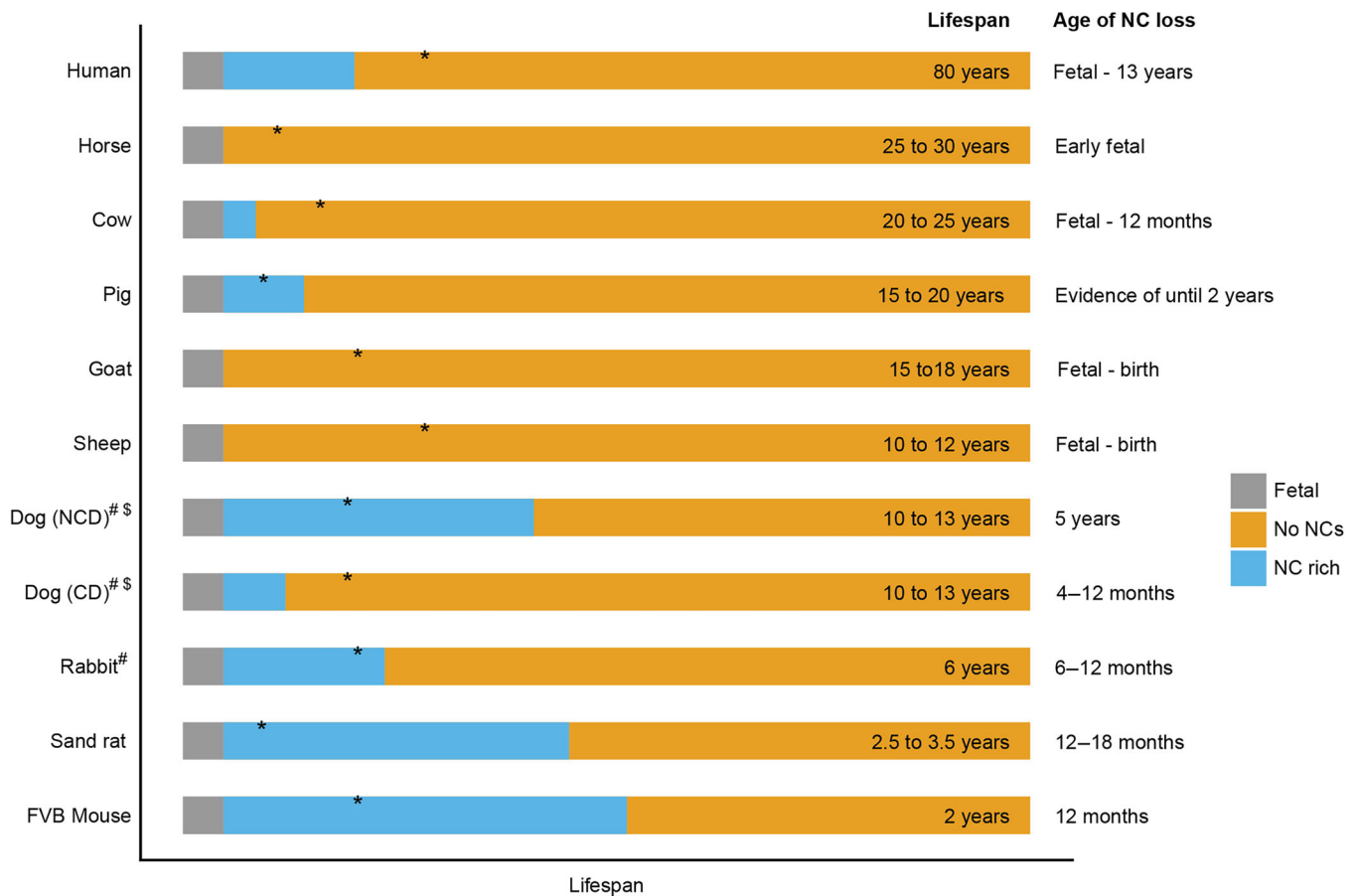
FIGURE 1 Comparison of the age at which notochordal cells (NCs) are lost from the intervertebral disc. Lifespan refers to approximate lifespan in captivity and can vary depending on environmental factors and other variables. *Time of skeletal maturity (human 20 years; horse: 2 years; cow: 2–3 years; pig: 12 months; goat: 2–3 years; sheep: 2–3 years; dog [NCD] and dog [CD]: 1–2 years; rabbit: 10 months; sand rat: 2 months; FVB mouse: 4 months). #Depends on breed and genetic background. $loss of NCs can depend on breed, genetic background, diet, obesity and injury. In the human, sheep and cow, NC loss begins at fetal stages, with NCs seen at birth. Horse discs in later stages of fetal development are devoid of NCs and none are seen at birth. NC, notochordal cells; CD, chondrodystrophic; NCD, nonchondrodystrophic (References: human[19,20,30–34]; horse[35]; goat[36]; sheep[21,29,37]; cow[22,23]; dog (CD)[24]; dog (NCD)[24,38]; rabbit[25,26,29]; sand rat[27]; FVB mouse[28]).
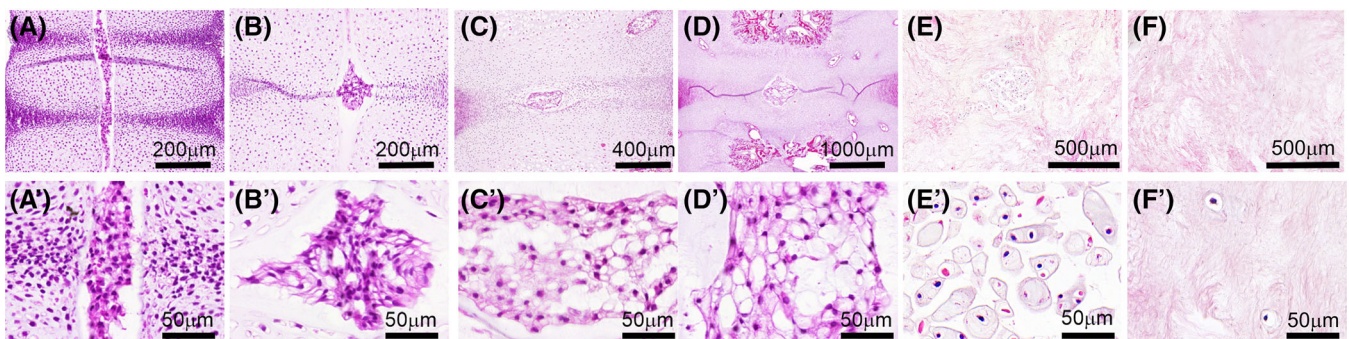
FIGURE 2 Notochordal cells within Human Intervertebral discs. Hematoxylin and eosin (H&E)-stained human intervertebral disc (IVD) tissue during development, shown at low and high magnification. Staining demonstrates large vacuolated morphology of cells within the developing nucleus pulposus (NP) region at (A) 7 weeks postconception (wpc), (B) 8.5 wpc, (C) 10 wpc, and (D) 17 wpc. (E) Retention of clusters of large cells within the nucleus pulposus (NP) region in a 10-year-old IVD, whereas (F) shows presence of only single, smaller mature NP cells within the NP region in a 17-year-old IVD.

disruption of normal phenotype.[67] To date, there is a lack of consensus for the extraction, numeration, in vitro culture, and characterization of NCs. Discrepancies observed in results could be explained by species-specific differences; however, large variations in methodological approaches are likely a major contributor. Thus, this article aims to provide key recommendations and methodologies for NC isolation,
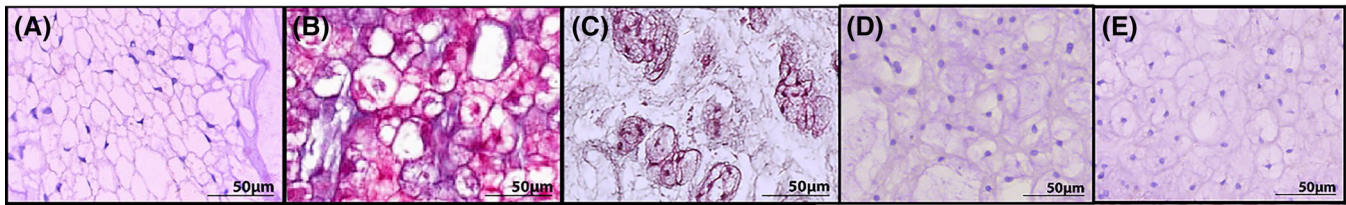
FIGURE 3   Notochordal cells are maintained in mature discs of mice (A), rats (b), rabbits (c), pigs (D), and some breeds of dogs (E). Images shown are from rodents and pigs <3 months of age, Dog image is from a mixed breed nonchondrodystrophic dog at 1 year of age. Hematoxylin and eosin-stained images, Scale bar = 50 μm.

numeration, in vitro manipulation, and characterization to support research into NC physiology and potential in regenerative therapies.

2 | RECOMMENDED METHODOLOGY FOR EXTRACTION OF NCS FROM THE NP OF MULTIPLE SPECIES

2.1 | Recommendations for dissection and aseptic isolation of IVD/NP tissue

NCs are regularly sourced from several species for in vitro investigations. These include small rodents such as mice and rats where lumbar and caudal (tail) discs are commonly used, however, caudal discs within mice are often preferred due to their higher numbers and ease of accessibility, whilst lumbar and caudal discs are often sourced from rats. However, given the size of rodent IVDs, isolation of pure NP tissue can be problematic and may require downstream fluorescent-activated cell sorting (FACS). Furthermore, generating sufficient cells for downstream analysis normally requires the pooling of discs. Large species such as NCD dogs (IVDs ∼20 mm depending on the breed), and pig (IVDs ∼30 mm at 3 months) provide large NP tissue sources which are easier to isolate. Within species which lose their NCs during development, that is, humans and CD dogs, fetal (7–25 weeks postconception [wpc] human) or young dog discs are sourced. Dependent on age of the donor, the separation of NP and AF may not be possible in the case of earlier stages of development, like pre segmentation spines, and thus FACS is recommended to ensure pure populations for study.

2.2 | Aseptic isolation of the spine/tail

This method outlines the procedure for dissecting the spine or tail (where appropriate) into intact spinal units. However, as some samples, for example, human samples obtained from early pregnancy terminations, are not always received intact, adaptation of this procedure may be required based on the state of the tissue on arrival. To ensure sterility external surfaces such as whole mice, rats, or pig tails/lumbar sections received from the animal house or external sources (i.e., abattoirs) remove first any debris and blood and clean with 70% ethanol (note: only use 70% ethanol if there are tissues external to the IVDs). For isolation of the spinal column of whole

cadavers where available, eviscerate and cutoff the limbs as close to the spine as possible and dissect the spinal column according to the procedure described in detail by Lee et al.,[68] For isolation of caudal discs a longitudinal incision along the dorsal side of the tail is performed to reveal the underlying tissues. Dissect away the skin, muscles, and other tissues exposing the IVDs, whereafter the exterior of the spine/tail is aseptically cleaned according to surgical protocol (two times with Hibiscrub [chlorhexidine gluconate 40 mg/mL]) via spraying with chlorhexidine for two times both followed by 3 min incubation.

2.3 | Dissection of the IVD from the spine/tail

For the isolation of IVDs from small specimens, for example, mouse, rat, young dogs, and human fetal samples, IVDs can be seen as bright white stripes between the more translucent vertebral bodies (Figure 4A). It is recommended to use a dissection microscope or binocular glasses to aid with the isolation of the IVD, starting at the most anterior disc. While holding the spine with the fine forceps, use for dissection a microsurgical knife (e.g., nr 11 scalpel knife for larger IVDs) or ophthalmic knives (Beaver® Optimum™ Knives) or an equivalent such as micro blades or a stab knife for small discs such as mouse caudal discs. The stab knife is recommended because discs from mice are very small ∼2 mm in diameter and the standard nos. 10/11 scalpel blades are too large and could lead to accidental cutting into the IVD, immediate depressurization of the NP and loss of the tissue (Figure 4B). Cut transversely along the plane of the anterior side of the disc, cutting off the vertebral body adjacent to it (Figure 4C). The disc/vertebral body boundary should be a natural line of least resistance. For younger donors (fetal <18 wpc in humans) where the AF and NP tissues have not undergone segmentation or the vertebrae are not entirely calcified, it may not be possible to isolate the NP tissue from surrounding tissues. In such a case the whole IVD is dissected, and a mixed population of cells isolated which may include NC, AF, and CEP cells which could then be separated using FACS (See section 2.5).

For older donors of NC-rich discs where the NP and AF tissues can easily be distinguished, the AF is seen as a white ring surrounding a translucent jelly-like NP (Figure 4C,D). For mouse discs use a pair of fine-curved forceps to "spoon out" but it is essential you do not pinch the NP tissue from the disc (Note: pinching via standard forceps application will traumatize the tissue and diminish cell viability; Figure 4Di) and place this onto the wall of an empty sterile Eppendorf tube on ice (Figure 4Ei).

FIGURE 4 Dissection of mouse and stillborn fetal human lumbar spine (21 weeks of gestation). (Ai) Mouse motion segment and (Aii) Human fetal L3, L4, and L5 vertebrae with intervertebral discs (IVDs) L3–L4 and L4–L5. (Bi) Ophthalmic knife and scalpel blade no 10 in comparison to whole mouse IVD demonstrating importance of use of ophthalmic or stab knife for extraction. (Bii) Whole fetal human IVD isolated for scale (1 cm in diameter). (C) The IVD should be excised by cutting at the junction between endplate and annulus fibrosus (AF) shown for mouse (Ci) and human (Cii) IVDs, noted within human fetal IVD end plates with blood vessels (white arrows) can be clearly seen. (D) The nucleus pulposus (NP) region is carefully removed from the IVD in the mouse (Di) this is spooned out with curved forceps, whilst human NP (Dii) can be carefully separated with a nr 11 scalpel blade separating out from the AF and EP tissue. (E) The tissue of the mouse IVD is placed carefully in the edge of an Eppendorf tube and (F) multiple NP tissue samples can be combined together for mouse (Fi) or individual NP tissue may be collected separately for human (Fii) dependent on size. The sample can further be processed by enzymatic digestion (G) prior to centrifugation and resuspension in resuspension media (H). (I) Mouse NP tissue shown pre digestion and (J) after digestion. Scale bar = 500 μm.
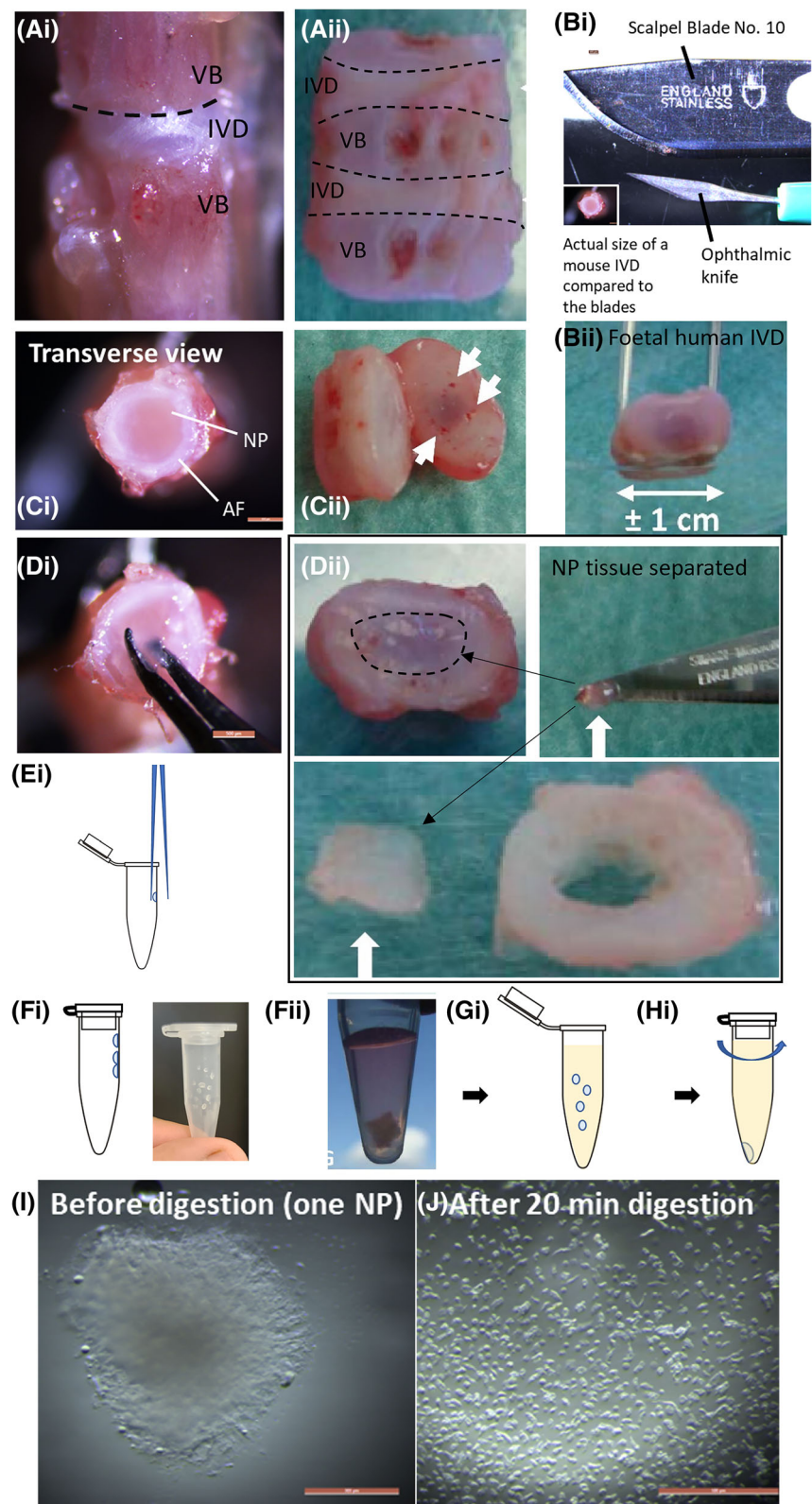
Whilst for larger discs such as young dog and fetal human (> ∼ 18 wpc) discs the NP and AF are separated manually using a curettes size A (World Precision Instruments, 501 773; Figure 4Dii). Mouse NCs retain high viability in a sealed empty tube on ice, or room temperature for up to 1 h. Collect multiple levels of the NP as required into the same tube to minimize variation of the digestion time (Figure 4F), and proceed to digestion of the tissue (Figure 4G,H). A freshly extracted mouse NP is observed as a clump of cells, but after 20 min digestion, are separated into single cells (Figure 4I,J). For larger IVD such as those from adult NCD dogs and pig IVDs, open the IVD space with a sterile no 22 blade at the border of

TABLE 1 Digestion protocols.

Tissue type	Digestion enzymes	Resuspension medium
Mouse tissue	20 min in HBSS with 850 U/mL Collagenase II and 2.6 U/mL Dispase	HBSS +2% BSA
Rat tissue	30 min with 7 U/mL pronase followed by 4 h digestion with 125 U/mL collagenase type II	αMEM +1% P/S
Young dog tissue	1 h in αMEM with 37.5 U/mL collagenase type II	αMEM +1% P/S + 2% BSA
Adult dog tissue (NCD)	30 min in αMEM with 7 U/mL pronase followed by 4 h with 125 U/mL collagenase type II	αMEM +1% P/S
Pig tissue	30 min in αMEM with 7 U/mL pronase followed by 4 h digestion with 125 U/mL collagenase type II	αMEM +1% P/S
Fetal human tissue	30 min in αMEM with 37.5 U/mL collagenase type II	αMEM +1% P/S

Note: For mouse, young dog, and fetal human NP extraction enzymatic digestion with pronase, higher concentrations of collagenase, or longer digestion times considerably decreases NC viability and is not necessary because of the loose extracellular matrix. Collagenase (Worthington, LS004177), Dispase (Worthington, LS02109), and Pronase (11 459 643 001, Roche Diagnostics), are filtered with a 0.22 μm membrane filter to ensure sterility, all incubations are performed at 37°C.
Abbreviations: αMEM, alpha modified eagles media; BSA, bovine serum albumin; HBSS, Hanks buffered saline solution; NC, notochordal cell; NCD, nonchondrodystrophic; NP, nucleus pulposus; P/S, Penicillin/Streptomycin.

the cranial endplate. Distract the IVD space with the aid of a Hohmann retractor (approaching the spinal canal from the adjacent opened IVD). Collect the gel-like NP with the aid of a curette and transfer into a 50 mL tube with 10–15 mL alpha modified egales media (αMEM) +1% penicillin/Streptomycin (P/S).

2.4 | Recommendations for digestion of NP tissue and NC extraction

Following dissection, the NP tissue is placed in a 50 mL tube with 10–15 mL αMEM +1% P/S, or 15 mL centrifuge tube for smaller discs (e.g., mouse) with 5 mL media. Centrifuge at 500 *g* for 5 min at room temperature (RT) and discard the supernatant. Of note is that contrary to degenerate NP tissue, NC-rich NP tissue is gel-like and generates a relatively loose pellet upon centrifugation requiring caution during removal of the supernatant. Incubate the collected NP tissue in digestion enzyme as per species specific methodology (Table 1). Place the tube on an orbital shaker at 37°C, 300 rpm during enzyme digestion. It is expected that after shorter term digestion periods, there will be a small number of remaining cell clumps, however, these can be readily dispersed by gently pipetting the cells with wide bore 1 mL tips. Centrifuge at 500 *g* for 5 min at RT and discard the supernatant. The expected outcome of this protocol is obtaining both single cells and clusters. Filter the sample over a 40-μm cell strainer (the filtered solution will contain the nonclustered cells). Collect the NC clusters by washing the filter upside down with resuspension media (Table 1). After centrifugation at 500 *g* for 5 min, resuspend the cells in αMEM with 1% P/S and count (see Numeration section). The <40-μm fraction will contain single cells and smaller clusters, whereas the >40 μm fraction will contain the larger clusters of predominantly NC-like cells with vacuoles, 25–85 μm in diameter.

Of note, mouse NC digestion was particularly sensitive to digestion time, with an increase of digestion time from 20 to 30 min resulting in lower cell viability (Figure S1). Furthermore, the addition of 2% vol/vol bovine serum albumin in hanks buffered saline solution (HBSS)

during digestion improved cell viability compared with HBSS alone (Figure S1), which has also been found for puppy NC isolation, whilst this was not necessary for NC isolation from other species. A small study was conducted to compare harvested NCs from pig spines versus tail IVD which are often waste material from the meat industry. More NP tissue was extracted from pig spines than tail IVDs in line with the larger IVDs within the spine (Figure S2). However, no difference was observed in the total number of NCs harvested per disc (Figure S2) with greater number of NCs per gram of tissue in NP tissue taken from the smaller tail IVDs compared with larger spine IVDs. Furthermore, isolation from tails was experienced as simpler; IVD exposure was more straightforward than spines where collection is hampered by the facet joints and transverse processes. These results provide the opportunity to refine and reduce waste material, as tails can be sourced from waste material from the meat industry whilst the use of spines often wastes the associated meat joints, this is in line with 3Rs (replace, reduce, refine).[69,70]

2.5 | Digestion of whole IVD tissue where NP isolation is not possible (e.g., human fetal IVDs [<18 wpc])

The enzyme concentration and centrifugation steps reported in this section were designed to rapidly isolate cells and maximize the number of cells collected for RNA/protein extraction, thus further optimization may be required to ensure high cell viability (see section 2.4 as a guide).

Transfer the IVD to a petri dish containing PBS to remove blood cells from the sample, which may contaminate the collected NC population during FACS. Using fine forceps (scoop do not pinch), transfer the IVDs to 50 mL Falcon tube containing 15 mL 62.5 U/mL Collagenase Type II (Gibco®, 17 101–015) and 150 U/mL type I-S hyaluronidase (Sigma-Aldrich®, H3506) in αMEM media containing 1% antibiotic/antimycotic solution. Incubate on an orbital shaker at 37°C for 2 h. Centrifuge at 500 *g* for 5 mins at RT and discard the

supernatant. Suspend the cell-pellet in a prewarmed nonenzymatic cell dissociation solution (e.g., Sigma, C1419) and incubate on the orbital shaker at 37°C for 10 min to help dissociate further cell aggregates. Centrifuge at 2000 g for 5 min at RT and discard the supernatant. Resuspend in 1 mL sterile PBS and pipette gently to disperse the pellet. Thereafter, the cells are strained to remove cell aggregates or debris that would interfere with downstream FACS with 50 μm cup type Filcon® (BD Biosciences, 340 629). The Filcon® cup is prewet with 500 μL PBS, before the application of the 1 mL cell suspension. Following washing with 500 μL PBS, the cell suspension is centrifuged at 500 g for 5 min at 4°C and FACS can be conducted as reported previously.[45] The notochordal population is typically 5%–20% of total viable cells (NC, AF, and CEP cells) from the digested IVD.[45]

2.6 | Calculating cell number

The characteristics of NCs contribute to creating an increasingly difficult cell type to count, due to the large cytoplasmic vacuoles, freshly isolated NCs range from 10 to 40 μm in diameter[29,71] and NCs exist as a network of large cell clusters, containing 10–400 cells[64,72]; with multiple tight cell-to-cell adhesions.[73–75] Furthermore, these large cell clusters are surrounded by a thin layer of matrix (notochordal sheath) which physically separates one cluster from another.[73] Whilst several techniques and specialist cell counters have been developed to count clusters of cells,[76] they often fail to accurately enumerate single cells. In order, to gain the most efficient and reliable cell count, the cell clusters must be dissociated.

The customary methods of dissociation include using enzymatic approaches, such as trypsin, TrypLE, dispase, and accutase[49,77–82]; and/or mechanical approaches, such as filters, chopping techniques, microfluidic devices, and various ways of pipetting.[83] Jager et al.[83] compared different ways of dissociating human induced pluripotent stem cells, which are grown in large clusters (17 000 μm^2) if left untreated and therefore difficult to dissociate without cell loss.[77] They demonstrated that none of the mechanical methods of dissociation produced single cells or small cell clusters. Whereas the combination of TrypLE followed by trypsin/EDTA digestion produced dissociated samples where the individual cells could be easily distinguishable.[83]

Within this study, enumeration methods were investigated to enable efficient and reliable dissociation of NC clusters which has not previously been reported. A comparison of dissociation methodologies was investigated to determine influence on cell count, viability, and resulting diameter, utilizing an automated cell counter (NucleoCounter® NC-200™ [Chemometec, Gydevang, Denmark]). Freshly extracted NC clusters were harvested from a single donor pig spine, as described above, and 500 μL of NC cell suspension in αMEM +1% P/S (allowing for triplicate repeats) was used to undergo different cell dissociation reagents. Dissociation reagents included either (a) solution 10 lysis buffer (Chemometec); (b) Solution A100 and B (Chemometec); (c) Accutase (Sigma); (d) Trypsin/EDTA (Thermofisher, Gibco); (e) TrypLE (Thermofisher, Gibco); and finally (f) TryLE followed by Trypsin. For solution 10 lysis buffer method; 100 μL NC

suspension was directly added to 100 μL solution 10 lysis buffer (Chemometec). The Solution A100 and B methods were conducted by taking 100 μL NC suspension sample and directly adding Solution A100 lysis buffer (Chemometec), leaving the NC sample in 100 μL solution A100 for 0, 2, 5, and 10 min, before next adding 100 μL of solution B stabilizing buffer (Chemometec). The Accutase method involved adding 1 mL of accutase (Sigma) directly to 100 μL of NC cell suspension, incubated for 10 min at 37°C, prior to being centrifuged for 1 min at 400 g and resuspended in 1 mL αMEM +1% P/S. Trypsin/EDTA method involved adding 1 mL of Trypsin/EDTA (Thermofisher) directly to 100 μL of NC cell suspension, incubated for 10 min at 37°C, prior to being centrifuged for 1 min at 400 g and resuspended in 1 mL αMEM +1% P/S. Finally, TrypLE method also involved adding 1 mL of TrypLE (Thermofisher) directly to 100 μL of NC cell suspension, incubated for 10 min at 37°C, prior to being centrifuged for 1 min at 400 g and resuspended in 1 mL αMEM +1% P/S. After dissociating NC suspension was counted using the Nucleo-Counter® NC-200™.

A combined analysis of images captured and the cell count (Figure S3) demonstrated that solution A100 and B could produce accurate cell counts and displayed visible NC dissociation, with the observation of single cells, whilst accutase, Trypsin, TrypLE, and TrypLE + trypsin resulted in reduced cell counts (Figure S3). Repeatability, linearity and range was further tested with the Solution A100 and B. The method of dissociating NC clusters with Solution A100 followed by the stabilizing solution B buffer after 2 min, showed the most promising method of dissociation (Figure 5). Prolonged incubation of NC clusters in solution A100 for more than 2 min caused the release of "sticky" DNA from neighboring dying cells causing the observation of cell clumps (Figure 5), whilst addition of solution A100 followed by immediate analysis (0 min) did not dissociate NCs into single cells (Figure 5). Linearity studies demonstrated excellent linearity with a slope of 1.047 and R2 of 0.9979 compared with expected cell number (Figure 5). Thus, the recommended methodology for numeration is to add 100 μL of solution A100 lysis buffer (Chemometec) to 100 μL of cell suspension, and incubate for 2 min at RT. Following incubation 100 μL of stabilizing buffer (Solution B [Chemometec]) should be added. The resulting cell suspension with solution A100 and B is then loaded into the Via1-Cassette™ (Chemometec) and read with the NucleoCounter® NC-200™ to provide a NC count allowing for the 1 in 3 dilution of the original cell suspension due to addition of 1:1:1 cell suspension, Solution A100, and Solution B. If a NucleoCounter® is not available we would recommend following the dissociation method described above and proceed to simple cell count using a manual hemocytometer, whilst automatic counting decreases subjective counting and user error and increases throughput.

3 | CRYOPRESERVATION OF NCS

Cryopreservation of NCs in the presence of a cryoprotective agent comes with anecdotal challenges in achieving a high cell viability after thawing. During freezing, the intracellular water molecules can form
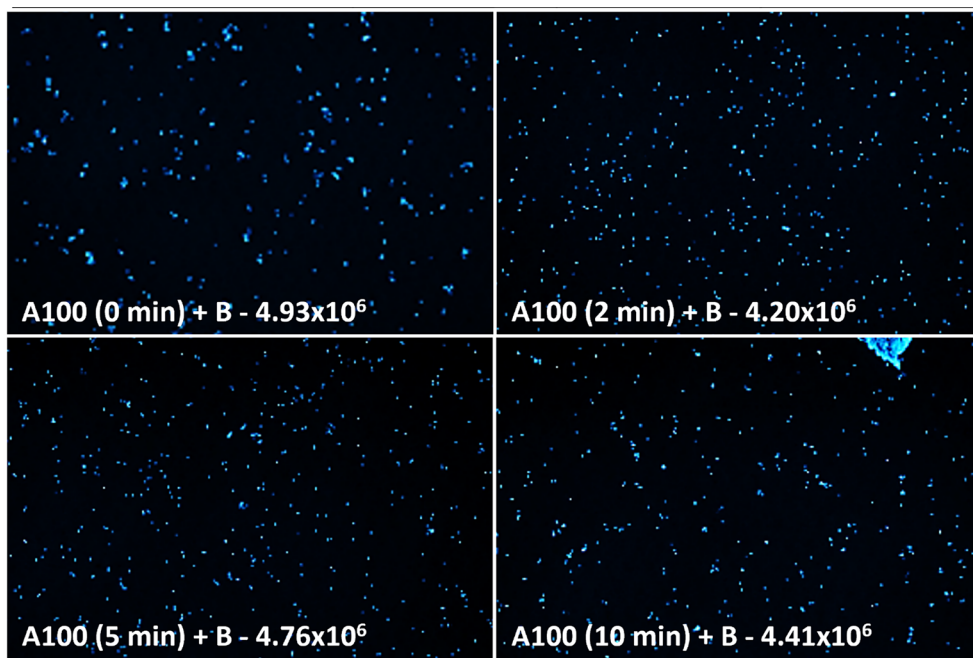
FIGURE 5 Numeration methodology for notochordal cells (NCs) using Chemometec nucleocounter. Dissociation of NCs was performed using solution A100 lysis buffer for 0, 2, 5, or 10 min prior to addition of Solution B stabilizing buffer. Similar total cell counts were observed; however, at 0 min cell clusters were still present and if A100 was left on for 10 min larger clumps could be visualized. Thus, a 2-min incubation time was selected for further linearity testing. Linearity studies comparing expected cell number via serial dilution was confirmed with a slope of 1.047 and R^2 of 0.9979.
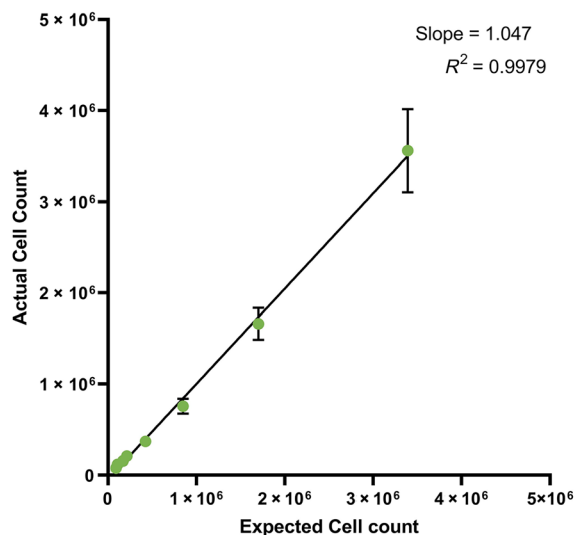
ice crystals which can damage cell membranes and other organelles and result in cell death. Cryoprotective agents prevent the formation of these ice crystals,[84] nonetheless the effectiveness therefore may depend on the cell type. Mature NPCs have been shown to maintain high viability after cryopreservation with the use of dimethylsulfoxide (DMSO) and fetal calf serum (FCS) as cryoprotective agents,[85,86] and is the most utilized methodology in the spine field.[87] Yet, there is little information on the cryopreservation of NCs. One of the key challenges relates to the NC vacuoles thought to contain a high-water content making NCs more susceptible to the formation of ice crystals in comparison to nonvacuolated NPCs.[88] In addition, NCs are present in cell clusters, which could prevent the cryoprotective agents from entering cells within the center of large cell clusters.

To determine whether NCs could be cryopreserved and the optimal method to maintain viability and phenotype, different types and concentrations of cryoprotective agents were examined. The NCs were extracted from 10-week-old pig spines ($n = 3$) and cryopreserved in (a) 20% vol/vol FCS in αMEM with different percentages of DMSO (0%, 5%, 10%, or 20%), (b) 10% glycerol +90% FCS, or (c) using a commercially available cryoprotective agent: CryoStor® CS10 (07930, Stemcell Technologies). Freshly isolated NCs were cryopreserved in a precooled (4°C) Mr. Frosty™ freezing container which was put in −80°C overnight. Sixteen hours thereafter, the NCs were transferred to liquid nitrogen (−196°C). After 1 week, the NCs were thawed in a water bath (37°C) and the number and viability of the NCs were determined using the NucleoCounter® NC-200™.

Since NCs can be compared with oocytes because of their large size and high-water content and oocytes are successfully cryopreserved with in-straw vitrification-dilution (ISVD), this method was also tested for NC cryopreservation. Straws were prepared as described by Inaba et al.,[89] As handling medium, αMEM was used and as a diluent solution αMEM +0.5 M sucrose. Both single NCs (<40 μm fraction) and clusters (>40 μm fraction) were resuspended in 1.6 M ethylene glycol and incubated for 5–15 min at RT to adjust to the

isotonic volume. After centrifugation, the cells were resuspended in 75 μL vitrification solution (1 M sucrose +10% glycerol in αMEM).[90] Three times 25 μL cell suspension was taken up per straw, whereafter the straws were sealed using a heat-sealing machine. The straws were frozen directly in liquid nitrogen and stored at −196°C. The ISVD straws were thawed by holding them in the air for 5 s, whereafter they were swirled in a water bath (37°C, 8 s). The straws were placed in a vertical position for 1 min to mix the different contents, whereafter they were placed horizontally for 5 min to allow the cells to calibrate to their osmotic environment and temperature. Next, the sealed parts were cut off and the cells were transferred to empty tubes. After centrifugation (500 *g*, 5 min) cell viability was assessed using the NucleoCounter® NC-200™.

The viability of the NCs directly after extraction was high (>70%) (Figure 6), however, following cryopreservation low viability levels were seen (between 5% and 20% in all the different concentrations of DMSO and the 10% glycerol +90% FCS solution; Figure 6). IVSD cryopreservation failed to yield viable NCs after thawing.
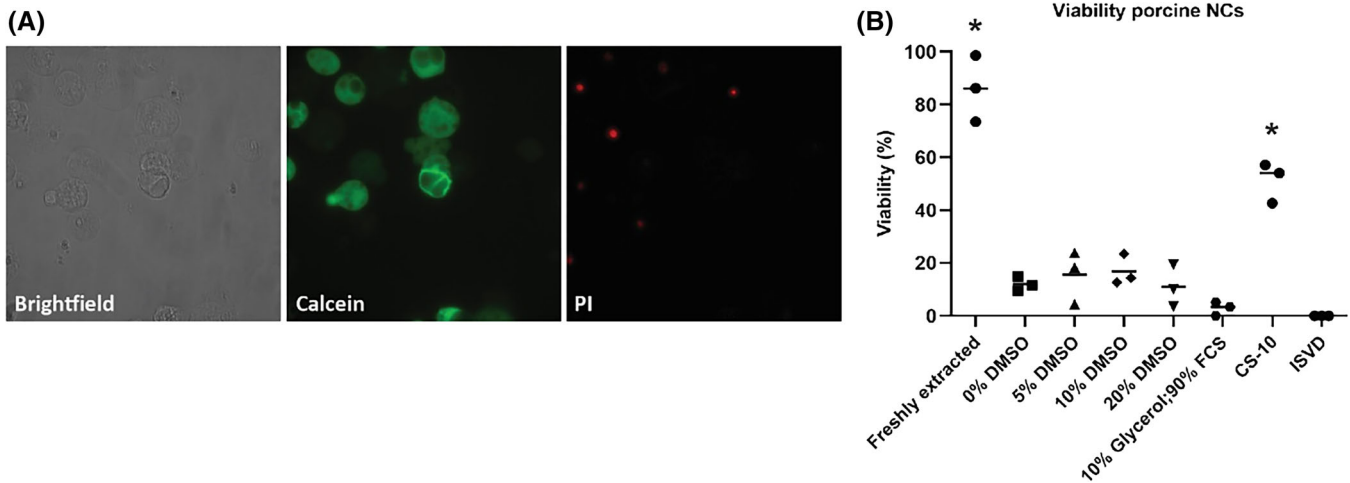
FIGURE 6 Viability of porcine notochordal cells (NCs) following cryopreservation testing: (A) Brightfield and Calcein-AM and propidium iodide (PI) staining images of freshly extracted porcine NCs. (B) Viability of NCs directly after extraction, after cryopreservation using different methods (*n* = 3 donors). *p < 0.05 from all other conditions except condition with same symbol. Statistics performed with IBM SPSS statistics, Mann–Whitney *U* test, corrected for multiple comparisons (Benjamini–Hochberg post hoc test). ISVD, in-straw vitrification-dilution.

FIGURE 7 Histological appearance of notochordal cell (NC) rich pig disc: NCs can be visualized clearly within a rich matrix of collagens and proteoglycans. NCs can be seen in large clusters of cells and display a vacuolated morphology. Which can be visualized with a variety of histology stains including hematoxylin and eosin (H&E), Safranin O-Fast green, Toluidine Blue, and Alcian Blue-Picrosirius Red.
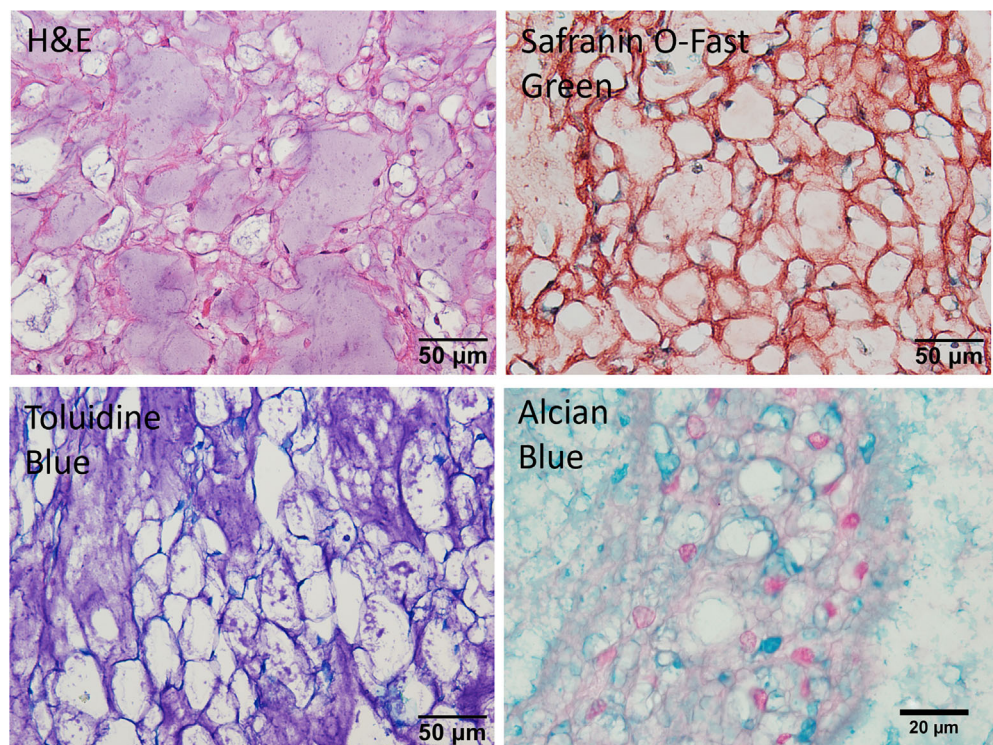
TABLE 2 Detection of key phenotypic markers in the embryonic notochord cells (eNC), notochordal cells (NCs), and the nonvacuolated nucleus pulposus cells (NPCs).

Marker gene	Description	eNC	NC	NPC	References
TBXT (T, Brachyury)	T-Box Transcription Factor T. Involved in the transcriptional regulation of genes required for mesoderm formation and differentiation				19,44,71,91–94
CAV1	Caveolin-1. Acts as a scaffolding protein within caveolar membranes				95–97
CD24	Cluster of differentiation 24. Cell surface sialoglycoprotein expressed at the surface of immune as well as epithelial, neural, and muscle cells				19,44
DSTYK/RIPK5	Dual serine/threonine and tyrosine protein kinase. NC Sheath protein				98
FOXA2	Forkhead box protein A2. Transcription factor involved in embryonic development; establishment of tissue-specific gene expression and regulation of gene expression in differentiated tissues				92
FOXF1	Transcription factor involved in embryonic development, establishment of tissue-specific gene expression and regulation of gene expression in differentiated tissues				19,99,100
FOXJ1	Forkhead Box J1. Transcription factor specifically required for the formation of motile cilia				101
KRT18	Keratin 18. Encodes the type I intermediate filament chain				19,34,44
KRT19	Keratin 19. Smallest known acidic cytokeratin specifically expressed in the periderm, the transiently superficial layer that envelopes the developing epidermis				19,44,91
LGALS3	Galectin 3. Encodes advanced Glycation End-Product Receptor				19,34,44,102
NOG	Noggin. Inhibitor of bone morphogenetic proteins (BMP) signaling which is required for growth and patterning of the neural tube and somite				103
NOTO	Notochord homeobox. Transcription regulator acting downstream of both FOXA2 and Brachyury during notochord development				13,92,104
SHH	Sonic Hedgehog Signaling Molecule. Encodes a protein that is instrumental in patterning the early embryo				42,91,92,103,105
SOX-5	SRY-Box Transcription Factor 5. Involved in the regulation of embryonic development and in the determination of the cell fate				17,106

TABLE 2 (Continued)

Marker gene	Description	eNC	NC	NPC	References
SOX-6	SRY-Box Transcription Factor 6. Involved in several developmental processes, including neurogenesis, chondrocytes differentiation and cartilage formation				17,106
SOX-9	SRY-Box Transcription Factor 9. Transcription factor involved in chondrocytes differentiation and skeletal development				92,106–111
ACAN	Aggrecan. Major proteoglycan component of the IVD extracellular matrix				42,75,102,110,112–114
AQP-6	Aquaporin 6. Encodes for a membrane proteins that functions as a water channel in cells. Shown to clearly highlight vacuoles in NCs				71
CA12	Carbonic Anhydrase 12. Member of the large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide				19
CDH2 (CD325, N-CADHERIN)	Cadherin 2. Encoded preproprotein is proteolytically processed to generate a calcium-dependent cell adhesion molecule which plays a role in the establishment of left-right asymmetry, development of the nervous system and the formation of cartilage and bone				94,115
COL2A1 and COL2A2	Collagen Type II Alpha 1 Chain and Collagen Type II Alpha 2 Chain				33,42,94,110,116
COL6A1	Collagen Type VI Alpha 1 Chain				32,42,113,117–119
GJA1 (CX43, Connexin-43)	Gap Junction Protein Alpha 1. Encoded protein is a component of gap junctions, which are composed of arrays of intercellular channels that provide a route for the diffusion of low molecular weight materials from cell to cell				112,115
GLUT-1 (SLC2A1)	Glucose Transporter Type 1/ Solute Carrier Family 2 Member 1. Encodes a major glucose transporter				120–122
HIF-1α	Hypoxia-inducible factor 1-alpha. Regulates genes that enable cell survival in a hypoxic environment, including those involved in glycolysis, angiogenesis, and expression of growth factors				99,121
KRT8	Keratin 8. Typically dimerizes with keratin 18 to form an intermediate filament in simple single-layered epithelial cells. Plays a role in maintaining cellular structural integrity and also functions in signal transduction and cellular differentiation				13,19
PAX-1	Paired Box 1. Involved in pattern formation during embryogenesis and may be essential for development of the vertebral column				19,99,106,108

(Continues)

TABLE 2 (Continued)

Marker gene	Description	eNC	NC	NPC	References
TEK (TIE2)	TEK Receptor Tyrosine Kinase. Encodes a protein that acts as cell-surface receptor for ANGPT1, ANGPT2 and ANGPT4. Regulates angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading, reorganization of the Actin cytoskeleton, but also maintenance of vascular quiescence				123–125

Note: Evidence for expression identified at protein level based on immunostains, reporter expression, mass spectrometry or FACS. Confirmed protein expression is indicated for the cells residing within the notochord (eNCs) and the core of the intervertebral disc (NCs and NPCs). Within individual species: ⬗, zebrafish; 🐁, nonchondrodystrophic dog; 🐕, chondrodystrophic dog; 🐭, mouse; 🐀, rat; 🐖, pig; 🐄, cow; 🧍 human. Blue icons indicate examples presented in the current article (Figures 8 and 9). Note that immunopositivity is largely dependent on the antibodies employed and tissue processing methods and the absence of expression may indicate a lack of data and technical difficulties in protein detection. In the absence of protein expression data, evidence from in situ hybridization indicates the localization of gene expression in their cellular environment (Boxed symbols). Gene descriptions refer to data from https://www.genecards.org/ .

Interestingly, NCs cryopreserved in CS-10 had a much higher viability (50%–60%) than the NCs cryopreserved in all other cryoprotective agents (Figure 6). In conclusion, to date, all tested cryopreservation methods resulted in large loss in NC viability. CS-10 showed highest viability levels with only 50% NCs remaining viable, but as a commercial product the content of this solution is unknown and further work would be required to determine phenotype following cryopreservation. It is thus currently recommended that NCs are utilized directly from extraction and not cryopreserved.

3.1 | Characterizing the phenotype of NCs

Characterization of the phenotype of NCs residing in the IVD is essential to understand whether this phenotype can be maintained during culture. The morphological appearance of NCs, in particular the large vacuolated NCs, are distinctive and can be identified via numerous histological stains as described previously[68] and showcased in Figure 7.

Here, we describe potential markers which can be useful to investigate key phenotypic and functional features of NCs, and whilst these are not all specific to NC phenotype, together they form a picture of cell status (Table 2).[13,17,19,32–34,42,44,71,75,91–125] Cells that reside within the NP experience dynamic changes of gene transcription profiles throughout postnatal cell proliferation, differentiation, IVD growth, aging, and degeneration. The recent review by Bach et al.,[15] discussed potential markers which can be utilized to aid in the characterization of eNCs, NCs to NPCs highlighting key issues with identification of NC/NP-specific markers and differential results between studies. Whilst a number of key markers have been suggested to identify NC and NPCs, the evidence for NC-specific markers is currently lacking, with cross expression across multiple cell types (Table 2). Expression of certain previously proposed NP markers (SHH, TBXT, KRT18/19, CA12, CD24, HIF-1α, GLUT-1)[40] are lost in older mature NPCs and hence could be useful to separate NCs from mature NPCs. Considering that the NC-rich NP can be properly isolated, the use of these markers is not complicated by the fact that they are also expressed by other similar cell types such as articular chondrocytes.[99] Interestingly many markers which have been described have not yet been investigated at protein level within most/any species, demonstrating a need for further characterization (Table 2).

In this context, key factors involved in normal NC physiology provide useful functional characterization markers for NCs (Table 2). Furthermore, NCs are distinguished from the smaller mature NPCs by the presence of vacuoles and the formation of large cell clusters, which are surrounded by a rich ECM NC sheath and thus key markers for these characteristics could be useful additional factors. Although cellular clusters are also observed during human disc degeneration,[126–129] and thus additional markers in addition to clustering are essential to separate the juvenile NC and a cluster of NPCs from degenerate discs. The NC vacuoles express high levels of aquaporins (AQP) on the membrane,[71] with AQP-6 demonstrating high intensity, which could be utilized to aid in vacuole identification (Figure 8). Dual serine/threonine
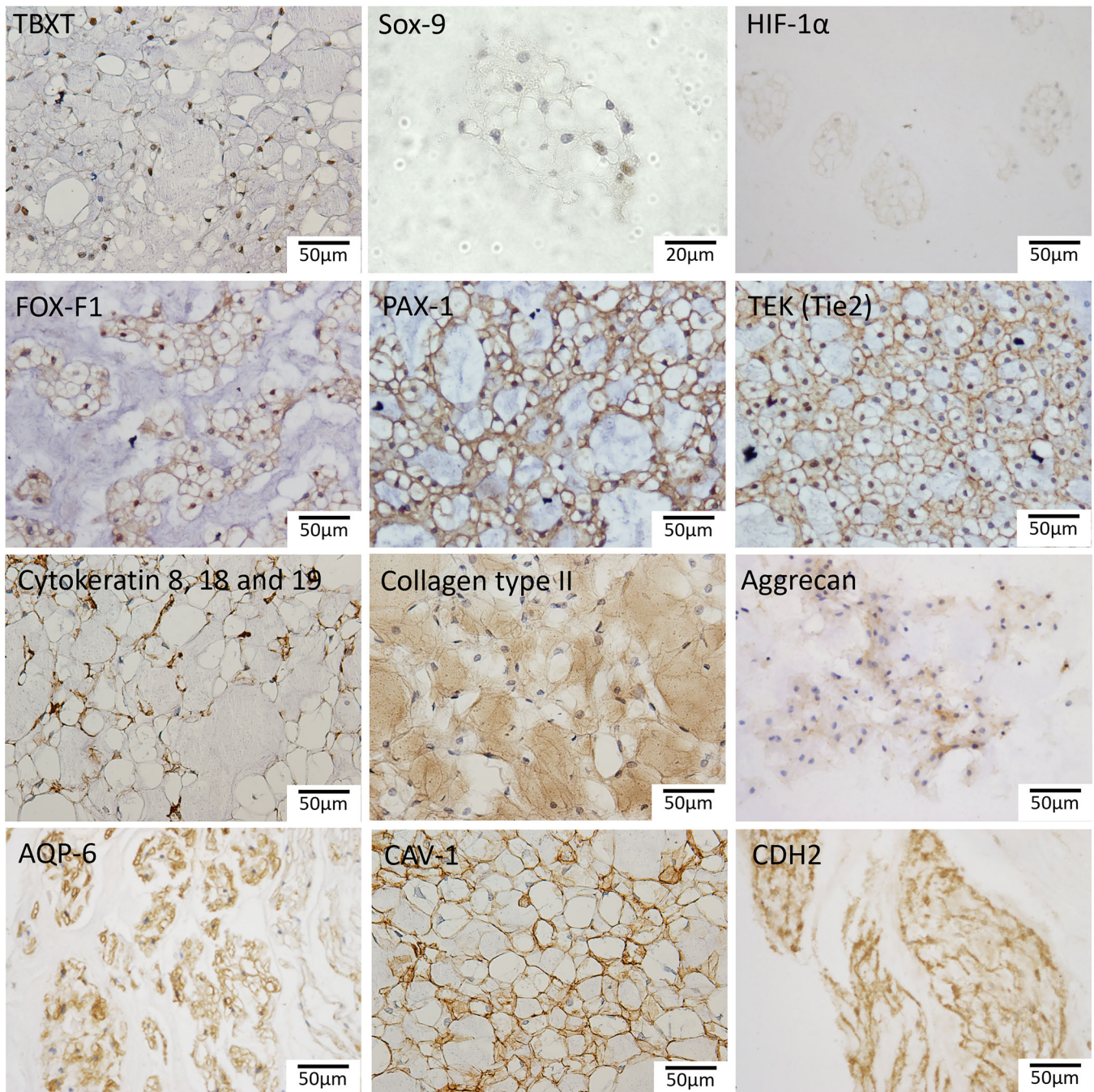
FIGURE 8 Immunohistochemical staining of notochordal cell (NC) rich pig and dog nucleus pulposus (NP) tissue: Immunohistochemistry was utilized to identify localization for potential characterization markers of NCs. TBXT, SOX9, HIF-1α, FOXF1, PAX-1, TEK (Tie 2), Pan Cytokeratin (8,18,19) Collagen type II, Aggrecan, CAV-1, and CDH2 were all observed within the NC rich regions of the NP of pig discs. AQP-6 staining was shown for dog discs. Scale bars = 50 μm except for SOX9 which is shown at higher magnification and 20 μm scale bar.

and tyrosine protein kinase (DSTYK) also known as receptor interacting protein 5 (RIPK5) is required for notochord vacuole biogenesis and integrity, DSTYK mutants in zebrafish causes scoliosis-like phenotype in fish, and overexpression leads to vacuole formation in sheath cells.[98,130–134] To date, limited studies have investigated the presence or role of RIPK5 within mammalian NCs, and therefore, its expression is investigated here within 3-month-old pig NCs. RIPK5 was identified

within porcine NCs, mainly around large NC clusters and thus could be a useful additional marker of NCs (Figure 9).

The "large NC clusters" are supported by multiple cell adhesion molecules, including gap junction proteins and cadherins. The presence of functional gap junctions anchored to the actin cytoskeleton, specifically connexin-43, has been shown in NC clusters from NCD dog NP.[73,74] Furthermore, cadherins which are membrane-spanning
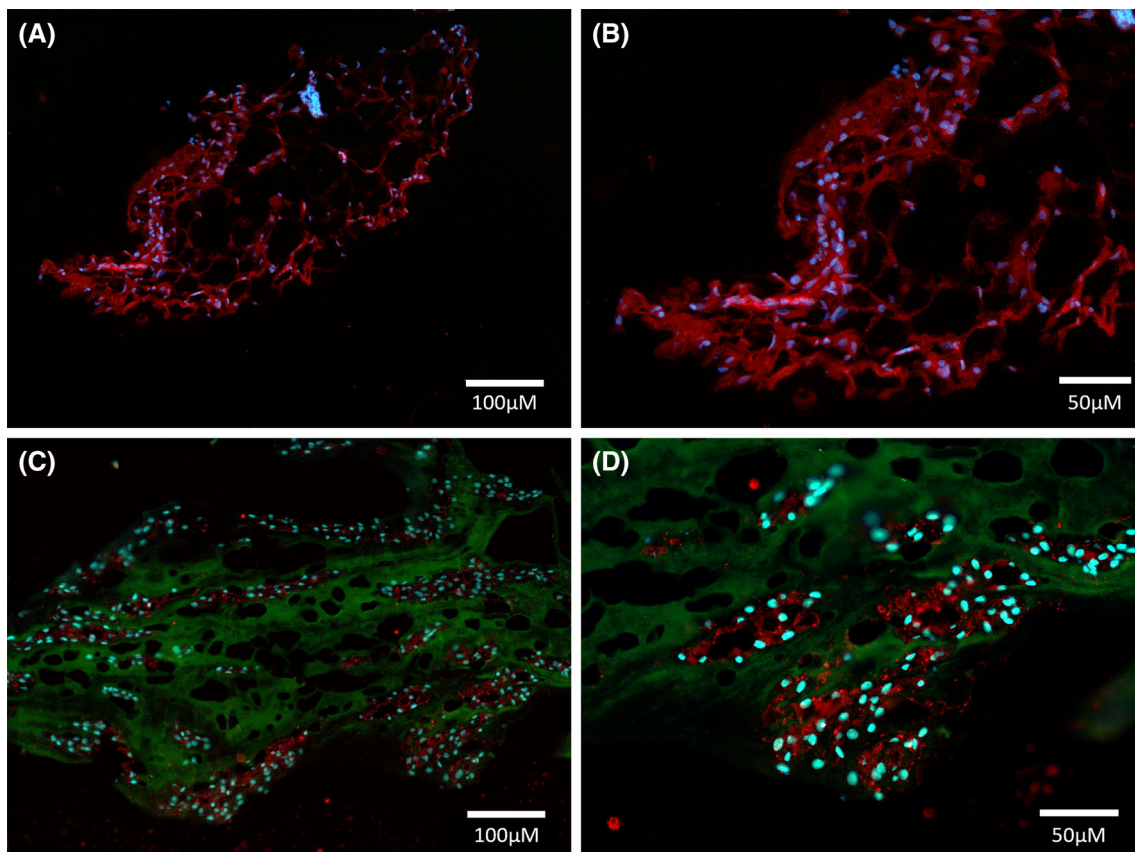
FIGURE 9 Immunofluorescence staining for notochordal cell (NC) rich pig NP tissue. Immunofluorescence staining for DSKY-5 (A,B), and costaining for AQP-6 (red) and collagen type II (green) (C,D). Scale bar as stated 100 or 50 μm.

macromolecular complexes responsible for cell–cell adhesion,[75,135] regulate the stability and cell contact formation. NC-rich NP tissues, including juvenile human discs and other animals, express high levels of CDH2(N-cadherin, CD325) with a decrease in CDH2 correlating with the disappearance of cell clustering during aging, as NCs are replaced by widely separated NPCs. Microarray and immunostaining have also implicated the membrane protein CAV1 (caveolin 1), an essential regulator of cell adhesion and migration, and is decreased during differentiation of NC to NPCs[52,95] and associated with progression of degeneration.[95]

The "NC sheath" has been shown to be highly important in the homeostasis of the developing spine within zebrafish, with scarce studies investigating this in mammals. The NC sheath is responsible for segmental patterning of the spine.[11,130] Structurally, the NC sheath is composed of three different layers that are comprised of laminin, fibrillin, fibronectin, proteoglycan, and collagen.[134] Studies that used zebrafish deficient in α1α4 or α1α5 chain of laminin,[131] the B1 and y1 chains of laminin-1,[134] Emilin-3,[132] Collagen 8[133] and Collagen 9α2[98,130] disrupt the sheath layer leading to a kinked spine and less vacuoles in NCs, which has also been linked to RIPK5 expression.

As a showcase, immunohistochemistry was utilized to determine the expression and localization of key proposed NC/NPC phenotypic markers within the NC-rich pig disc. Freshly extracted discs of juvenile pigs were formalin fixed and paraffin embedded, 4 μm sections were utilized for IHC (Table S1) using previously published IHC protocols.[136] NP tissue within the NC-rich pig discs were shown to express positive immunohistochemical staining for a number of potential NC markers including TBXT, SOX-9, HIF-1α, FOX-F1, PAX-1, TIE-2, and pan-cytokeratin (Figure 8). Furthermore, ECM markers collagen type II and aggrecan were identified both within the ECM and cellular expression (Figure 8). Vacuoles within NCs could be clearly seen using both AQP-6 and CAV1 markers (Figure 8), whilst CDH2 was also highly expressed (Figure 8). Immunofluorescence was also utilized to identify expression of DSTYK/RIPK5 within native porcine discs and was shown to be highly expressed by NCs, particularly located around large NC clusters (Figure 9), in a similar pattern to the NC sheath seen in zebrafish. Immunofluorescence also enables co-staining of cell and ECM markers, with the showcase demonstrated for AQP-6 and COL2 (Figure 9).

In conclusion, the characterization of NC-specific markers, conserved across species and of physiological relevance, that can be utilized to define stage-specific cell types found in the notochord and later in the NP from eNCs to NCs and then NPC is still subject to discussion by experts in the field and remains to be fully resolved (see review Bach et al.,[15]). Advances in "omic" technologies and in particular single cell or spatial profiling approaches hold promise to enable NC marker discovery and improve characterizing and benchmarking the NC and NPCs phenotypic changes across species during their life
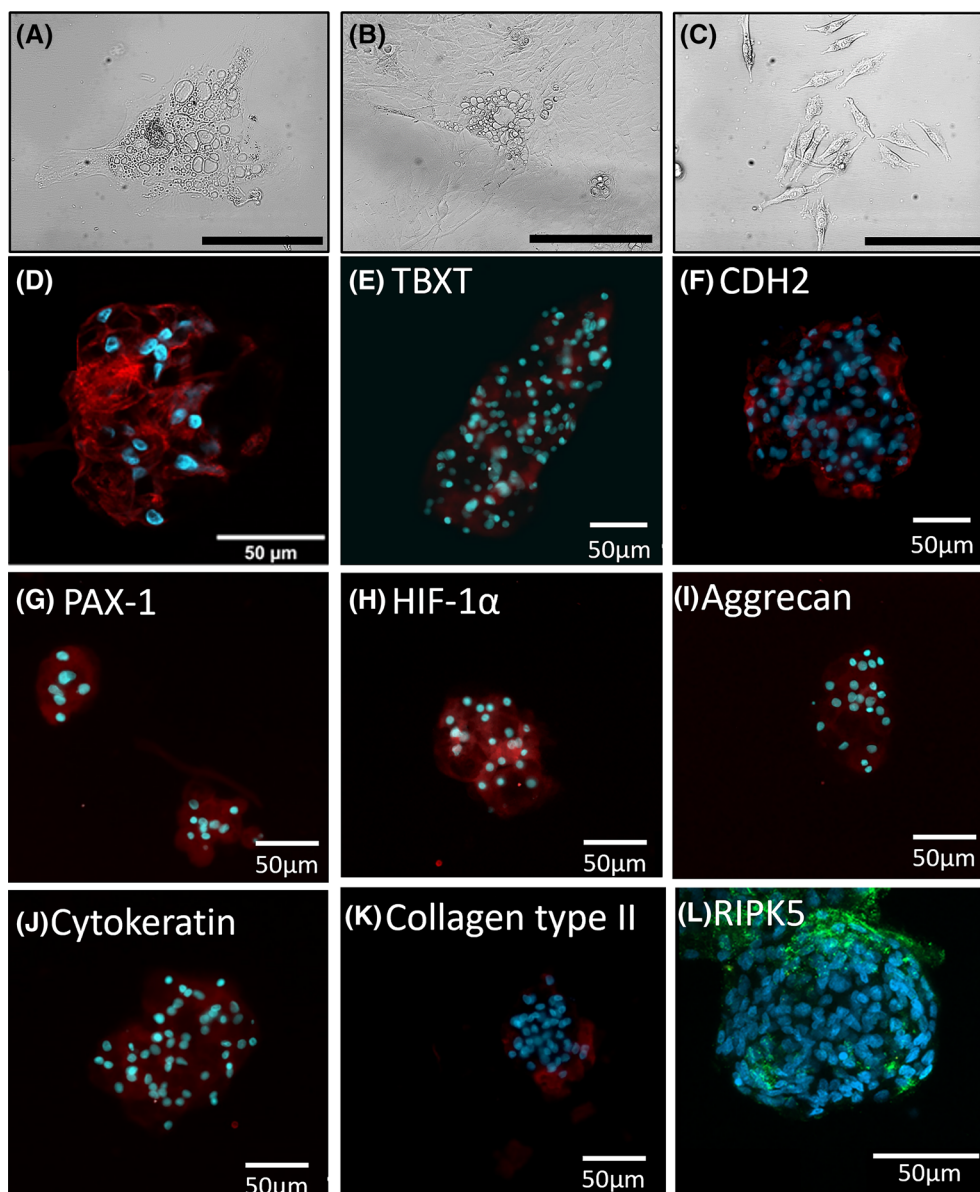
FIGURE 10   Notochordal cell (NC) morphology and phenotype within in vitro culture. (A–C) Monolayer culture and morphology of cells directly extracted from rat nucleus pulposus tissue. (A) Clusters of large, vacuolated NCs are the main cell type present during 2D in vitro culture 1 day after extraction from rat nucleus pulposus (NP) tissue, although some smaller, nonvacuolated cells are also present. (B) After 7 days in 2D in vitro culture, the number of clustered, vacuolated cells has reduced, whereas the number of smaller, nonvacuolated cells has increased. The remaining clusters of vacuolated cells are surrounded by smaller, nonvacuolated cells. (C) At 10 days postextraction, the clusters of large, vacuolated cells have almost completely been replaced by or differentiated into smaller, nonvacuolated cells. Scale bar 50 μm. (D) Rat NCs cultured in alginate in 400 mOSM/L αMEM maintained clusters and vacuolated morphology as shown by Phalloidin and DAPI staining following 1 day in culture. (E–L) Immunofluorescence staining for NC characterization markers in rat NCs cultured in alginate for 4 days. (E) TBXT, (F) CDH2, (G) PAX-1, (H) HIF-1α, (I) Aggrecan, (J) Pan Cytokeratin (8,9,19), (K) Collagen type II. (L) DSKY-5 immunofluorescence staining in porcine NCs cultured in alginate for 14 days. Scale bars = 50 μm.

journey.[42,43] Based on current knowledge particularly evidence from immunopositivity and the expression profile of potential markers which can be deployed to characterize NCs (Table 2 and Figures 8 and 9). However, given that many of the markers are expressed through eNCs, NCs, and NPCs to discriminate between these cell types and to evidence NC phenotype coexpression at the level of single cells should be aimed using techniques such as coimmunostaining analysis.

3.2 | Cell expansion and culture whilst maintaining phenotype

The expansion and culture of freshly isolated vacuolated NCs, whilst also maintaining their in vivo-like phenotype, remains challenging. If cultured within a standard monolayer system, NCs rapidly lose their clustered, vacuolated phenotype between 6 and 28 days or between
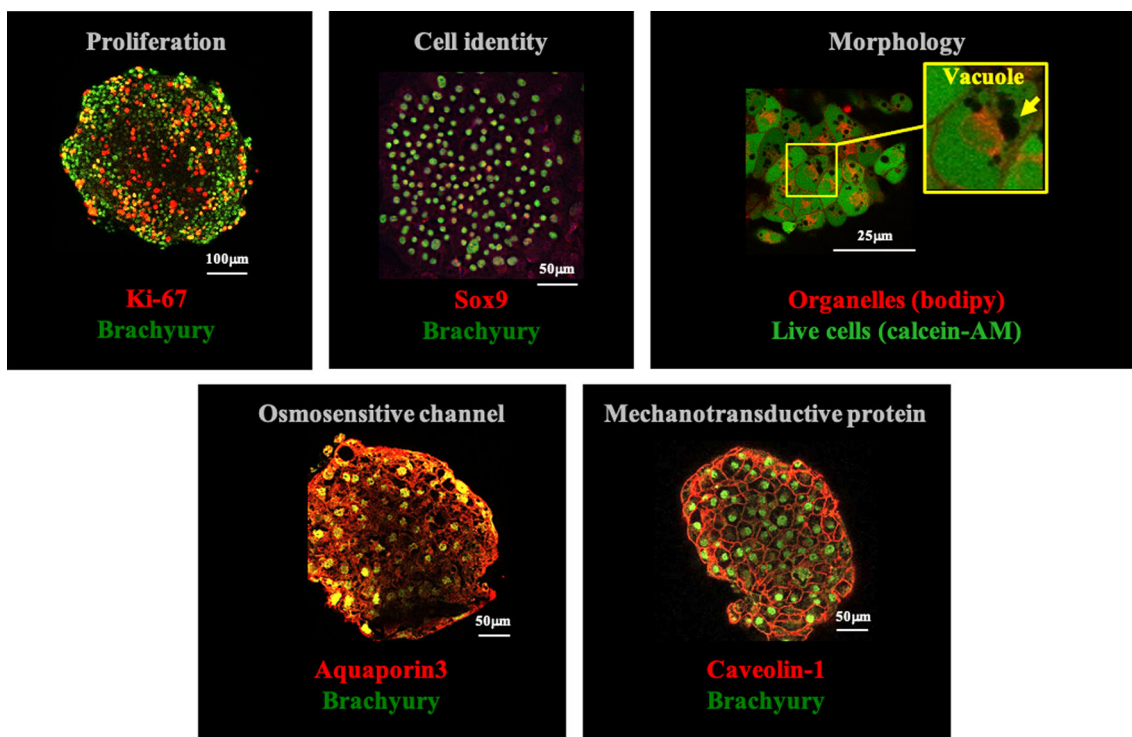
FIGURE 11 Micromass culture of mouse notochordal cell (NC) Immunofluorescence co-localization: Immunofluorescence images showing Ki-67+/Brachyury+ proliferative NCs, coexpressing SOX9+/Brachyury+, AQUAPORIN3+/Brachyury+, and Caveolin-1+/Brachyury+ NC-specific markers, respectively and showing vacuoles within the cytoplasm where the calcein-AM fluorophore is excluded (yellow arrowhead).

1 and 3 passages, and present morphologically as smaller, singular, nonvacuolated cells (Figure 10), a phenomenon reported across multiple species.[52,55,58,66] During this change in morphology, it has been observed that the expression of NC markers TBXT, KRT8/19, is decreased indicating that the smaller, nonvacuolated cells are phenotypically distinct from the originally extracted vacuolated NCs.[55,58] In addition, vacuolated cells are quickly outcompeted by small, nonvacuolated cells in monolayer culture, as their growth rate is significantly slower.[55,65]

Therefore, specialized techniques have been developed which utilize several environmental cues, for the retention of the vacuolated NC phenotype during in vitro culture and expansion. It has been well established that 3D alginate bead culture helps to maintain the in vivo phenotype of mature nonvacuolated cells.[67] When vacuolated cells are cultured within alginate beads viability and culture duration were increased, their clustered, vacuolated morphology was retained, when compared with monolayer culture, with most studies to date using αMEM media.[55,57,59,65] This indicates 3D alginate bead culture is an appropriate method that enables retention of native phenotype. In addition, alginate bead culture under physioxic (2% pO_2)[55] and physiological osmolarity (400 mOsm/L in αMEM media),[64] increased vacuolated cell marker expression and clustering. Furthermore, there is evidence that the vacuolated cell phenotype can be retained under specific culture conditions in vitro. Initial short-term alginate culture caused some loss of phenotype, yet this was restored after 28 days in culture, indicating the initial NC vacuolated

phenotype may be lost after isolation, but then at least partially recovered following extended 3D culture under appropriate osmotic culture conditions.[64]

3D culture systems such as alginate fail to enable expansion of NCs, thus alternative substrate surfaces together with environmental conditions have been investigated in the attempt to maintain phenotype whilst enabling expansion. Humphreys et al.,[137] investigated retention of the vacuolated cell phenotype of pig NCs in 2D culture, with the use of cell culture substrate coatings, oxygen concentration, osmolarity, and surface stiffness. The greatest levels of cell adhesion and proliferation, morphology, and expression of NC phenotypic markers (CD24, KRT8, KRT18, KRT19, and TBXT) were observed with the use of laminin-521-coated surfaces with 0.5 kPa stiffness and αMEM media, under 2% pO_2, 400 mOsm/kg culture conditions, where highest number of vacuolated NC cells (around 70%) were retained.[137] This indicates that the vacuolated NC phenotype can also be maintained under specific 2D culture conditions, where at least some population doubling is possible.[137] However, culture within such conditions can be difficult to obtain across laboratories and thus where proliferation of NCs is not required we recommend that clusters of isolated NCs are maintained as clusters and cultured within alginate beads in αMEM media, under physiological O_2 concentration (1%–5%) and osmolarity (400 mOsm/L), in FCS free media such as that described for alginate culture of NPCs with the inclusion of insulin-transferrin-selenium, Albumax and L-Proline,[87] as this enables the retention of their in vivo morphology

(Figure 10) and maintained the expression of NC phenotypic markers, including TBXT, CDH2, PAX1, HIF-1α, pan KRT, COL2, ACAN, AQP-6, and RIPK5 (Figure 10).

Recent micromass cultures have also been investigated for a simple and reproducible 3D culture model of NCs, to study basic biology and function, isolated from mouse immature NP tissue.[97] Culture conditions were optimized to enable growth of self-organized micromasses of NCs in suspension. This 3D model system successfully preserves the phenotype of mouse NCs, which was determined by maintenance of the intracytoplasmic vacuoles, the expression of NC characteristic markers (TBXT; SOX9) and the synthesis of proteins related to their function (CAV1; AQP3; Figure 11). However, this remains to be confirmed for other species and mature NCs.

4 | CONCLUSIONS

This article aims to provide key recommendations for the extraction of NCs from multiple species from mice to humans. Providing tips and tricks to harvest maximal viable cells to enable downstream studies and analysis with differential methodology particularly for fetal tissues. Considerations for numeration of NCs are provided with a recommended methodology which enables accurate counting despite the challenges of the tight NC clusters. The issues associated with cryopreservation of these cells are discussed and further work is required to identify whether these cells can be cryopreserved. Recommendations are provided for NC characterization: whilst to date no marker is specific to NCs, by utilizing a phenotypic panel approach their characterization is possible. New insights in phenotypic markers using single-cell transcriptomics, which many groups are currently working on, will in the future provide for improved characterization methodologies to further understand these fascinating cells. Together we hope that this article will provide a road map for in vitro studies using NCs derived from a range of commonly utilized species, which can enable acceleration of research.

AUTHOR CONTRIBUTIONS

Anne Camus, Marianna A. Tryfonidou, and Christine L. Le Maitre, contributed to conception of the study. Rebecca J. Williams, Lisanne T. Laagland, Frances C. Bach, Anne Camus, Marianna A. Tryfonidou, and Christine L. Le Maitre, contributed to the design of the study. Rebecca J. Williams, Lisanne T. Laagland, Frances C. Bach, Lizzy Ward, Shaghayegh Basatvat, Wilson Chan, Joseph W. Snuggs, and Lily Paillat contributed to acquisition of laboratory data (Rebecca J. Williams: Porcine and Rat isolation, numeration studies, characterization, and culture; Lisanne T. Laagland: Canine and porcine isolation and characterization; Frances C. Bach: human, canine, and porcine isolation; Lizzy Ward: human isolation; Shaghayegh Basatvat: porcine isolation; Wilson Chan: mouse isolation; Joseph W. Snuggs: rat isolation, monolayer culture, and characterization; Lily Paillat: mouse isolation). Rebecca J. Williams, Lisanne T. Laagland, Frances C. Bach, Shaghayegh Basatvat, Stephen M. Richardson, Wilson Chan, Joseph W. Snuggs, Lily Paillat, Marianna A. Tryfonidou, and Christine L. Le Maitre performed data analysis. Rebecca J. Williams, Lisanne T. Laagland, Frances C. Bach, Lizzy Ward, Wilson Chan, Vivian Tam, Adel Medzikovic, Lily Paillat, Joseph W. Snuggs, Deepani W. Poramba-Liyanage, Judith A. Hoyland, Danny Chan, Anne Camus, Stephen M. Richardson, Marianna A. Tryfonidou, and Christine L. Le Maitre contributed to interpretation of the data. Rebecca J. Williams, Lisanne T. Laagland, Frances C. Bach, Lizzy Ward, Wilson Chan, Vivian Tam, Adel Medzikovic, Shaghayegh Basatvat, Nicolas Vedrenne, Joseph W. Snuggs, Deepani W. Poramba-Liyanage, Danny Chan, Anne Camus, Marianna A. Tryfonidou, and Christine L. Le Maitre drafted the article. All authors critically revised the article for intellectual content. All authors approved the final version and agree to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

The authors have no relevant conflicts of interest to declare in relation to this article.

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SUPPORTING INFORMATION

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