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RESEARCH ARTICLE

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Harmonization and standardization of nucleus pulposus cell extraction and culture methods

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Abstract

Background: In vitro studies using nucleus pulposus (NP) cells are commonly used to investigate disc cell biology and pathogenesis, or to aid in the development of new therapies. However, lab-to-lab variability jeopardizes the much-needed progress in the field. Here, an international group of spine scientists collaborated to standardize extraction and expansion techniques for NP cells to reduce variability, improve comparability between labs and improve utilization of funding and resources.

Methods: The most commonly applied methods for NP cell extraction, expansion, and re-differentiation were identified using a questionnaire to research groups worldwide. NP cell extraction methods from rat, rabbit, pig, dog, cow, and human NP tissue were experimentally assessed. Expansion and re-differentiation media and techniques were also investigated.

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Results: Recommended protocols are provided for extraction, expansion, and re-differentiation of NP cells from common species utilized for NP cell culture.

Conclusions: This international, multilab and multispecies study identified cell extraction methods for greater cell yield and fewer gene expression changes by applying species-specific pronase usage, 60–100 U/ml collagenase for shorter durations. Recommendations for NP cell expansion, passage number, and many factors driving successful cell culture in different species are also addressed to support harmonization, rigor, and cross-lab comparisons on NP cells worldwide.

KEYWORDS

culture, harmonization, in vitro, intervertebral, nucleus pulposus, standardization

1 | INTRODUCTION

Chronic back pain (BP) is the leading cause for disability worldwide and is a socioeconomic burden for modern societies.[1–3] Degeneration of intervertebral discs (IVD) has been associated with approximately 40% of chronic BP cases.[4] Although IVD degeneration (IDD) per se also occurs in nonsymptomatic individuals,[5] this is more common in older individuals.[6] IDD is characterized with a switch of anabolism to catabolism with increased extracellular matrix (ECM) degradation, IVD, and bone remodeling, loss of hydration, and altered spine biomechanics.[7] IDD is also accompanied by the release of proinflammatory cytokines from the native disc cells, induction of angiogenesis, and neoinnervation.[7,8] IDD is multifactorial involving aging, genetics, environmental factors, and mechanical loading, yet the pathophysiology is not completely understood.[7,9] Treatment strategies are under development which target IDD, such as gene-, cell-, and biomaterial-based approaches.[10–16]

The IVD is situated between two vertebrae and consists of a soft, gelatinous central nucleus pulposus (NP), which is surrounded by the annulus fibrosus (AF), and cartilaginous endplates (CEPs), which provide permeable connections to the bony vertebrae allowing for nutrient supply to the IVD. Each region is populated by unique cell populations. The main function of the NP is to absorb and distribute mechanical load along the spinal column.[17] Therefore, the NP is composed of specific ECM components such as proteoglycans which retain water due to their negatively charged side chains attracting cations, allowing for the maintenance of a highly hydrated state and high swelling pressure. Owing to the IVD's largely avascular nature,[18] anaerobic metabolism by NP cells in a low oxygen microenvironment generates high levels of lactic acid leading to low pH, which further decreases during degeneration.[19,20] IDD is believed to initiate in the NP and propagate outwards.[21]

In vitro studies commonly utilize IVD cells to investigate disc cell biology, and pathogenesis of IDD, to shed light on specific molecular pathways or components within a controlled environment, or to aid in the development of new therapies, such as the possibility to use IVD cells for cell therapy and tissue engineering products. These are promising treatment options for progressive IDD, because the IVD has poor intrinsic healing capacity and early clinical trials delivering cells to the IVD have shown promise for improving healing.[14,22–25] The rationale behind delivering exogenous cells to the degenerated IVD is that delivered cells can directly synthesize new ECM, stimulate endogenous cells to synthesize ECM or secrete modulatory signals to reduce catabolism.[24,26] Though encouraging, there remain numerous challenges to overcome before cell therapies can be evaluated as a safe and effective clinical practice for IDD.[14,27,28] For example, there is no clear consensus on the optimal cell source[14,29] or dosage for IVD cell delivery.[30,31] The lack of consensus can be attributed in part to variability in the literature, which stems from differences in cell isolation and culture methods. This heterogeneity makes conclusions less definitive and more difficult to compare across studies.

Lab-to-lab variability jeopardizes the much-needed progress in the field.[32] In fact, multiple methodologies for IVD cell isolation and growing conditions are utilized within publications across all species. With, no consensus on isolation and culture methods to yield the highest IVD cell numbers and maintain viability and phenotype as close to in vivo as possible. Harmonizing the methods of extracting and expanding IVD cells is crucial to overcome these challenges for fundamental cell biology and translational studies. In doing so, we expect reduced variability across studies and an improvement in the ability to make meaningful comparisons and thereby advance the field.

As a first step to develop consensus methodology for IVD cells, the present study focuses on developing recommendations for NP cell isolation and expansion methodology, and a redifferentiation methodology to mimic the in vivo phenotype. NP cells were selected as the focus of this first step as this was more commonly reported in the initial survey; which we hope will lead to future studies for AF, CEP cells, and consensus methodology for tissue explant cultures and organ culture methods to reduce lab-to-lab variation and increase reproducibility across the spine field.

2 | MATERIALS AND METHODS

2.1 | Experimental design

To harmonize standard procedures routinely used within the IVD research field, this study aimed to collect and analyze commonly used protocols for the isolation of NP cells from IVD tissue, as well as provide recommendations on a standard culture media used for monolayer expansion of NP cells, and a redifferentiation methodology to mimic the in vivo phenotype (Figure 1). The authors decided to focus this first phase review on the small mature NP cells, with the isolation and phenotypic maintenance of the vacuolated notochordal cells (NC) being the subject of a secondary study and article.*

In the first stage of this study, we collated and compared extraction, expansion, and re-differentiation methodology for NP cells from human and animal sources, with respondents reporting methods for mouse, rat, rabbit, pig, dog, and cow IVD tissue from research groups worldwide (Figure 1). In the second stage, the authors formed an expert review of methodology together with prior published method comparisons to identify commonalities for immediate standardization and recommendations. Areas where variance in methodology were reported were identified as differential factors worthy of experimental testing. In the third phase, key differential factors were tested experimentally to identify extraction methodology which obtained highest cell yield whilst maintaining key phenotypic factors seen in vivo.[33,34] Extraction methodologies were investigated from NP tissue of humans, cows, dogs, pigs, rabbits, and rats, based on the protocols supplied and through expert review of methodologies, investigating enzyme types, concentrations and combinations, and digestion duration. Mouse cell extraction methodology was not investigated further in this article as this was felt better placed in the companion article on NC extraction; furthermore, detailed protocols for mouse NP cells for

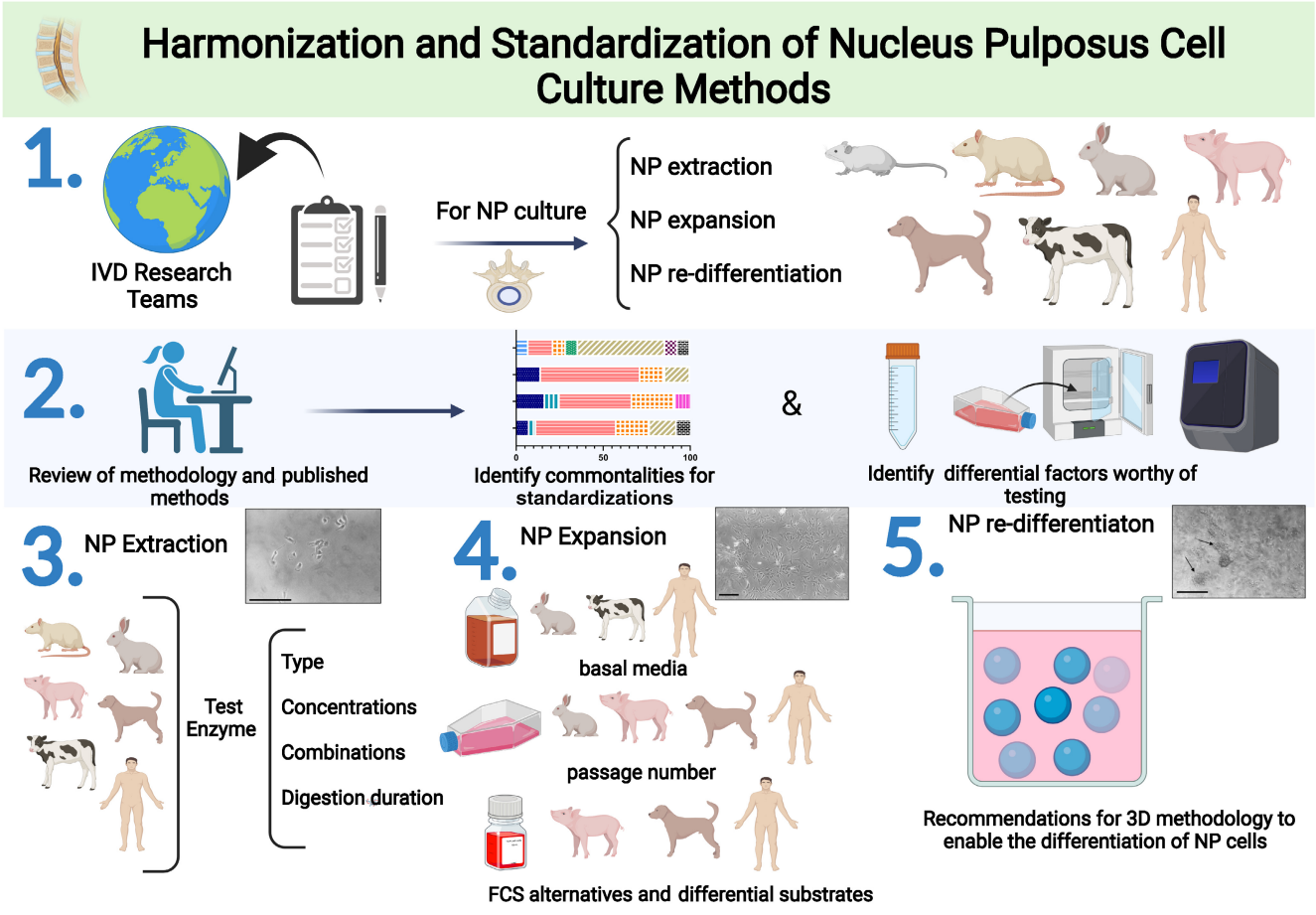


FIGURE 1 Experimental design: Initially (1), extraction, expansion and re differentiation methodology for nucleus pulposus (NP) cells from human and animal intervertebral disc (IVD) tissue from research groups worldwide were collated and compared. Second (2), the authors formed expert review of methodology together with prior published method comparisons to identify commonalities for immediate standardization and recommendations, and differential factors worthy of experimental testing. Third (3), differential factors to identify extraction methodology, to obtain highest cell yield while maintaining key phenotypic factors. Fourth (4), culture of NP cells was investigated to determine the effect of basal media (within rabbit, cow, and human), passage number (rabbit, pig, dog, and human), alternatives to fetal calf serum (FCS) and differential substrates (pig, dog, and human). Finally (5), this study makes recommendations for 3D culture methodology to enable the redifferentiation and investigated possible alternative culture media which can be utilized for 3D culture without the need for FCS to enable standardization across groups worldwide. Created with BioRender.com.

downstream RNA analysis have recently been published.[35] Furthermore, given the similarity with rat tissues, and the larger NP tissue available from rat discs, rat tissue was selected for through testing within this study. Fourthly, culture of NP cells was investigated to determine the effect of basal media (within rabbit, cows, and humans), alternatives to fetal calf serum (FCS) (within dogs and humans) and differential substrates (within dog, pigs, and human), selected species were identified here, to represent key species utilized for such studies as initial indications for recommendations. Fifthly, this study makes recommendations for 3D culture methodology to enable the redifferentiation of NP cells based on the groups' experiences and investigated possible alternative culture media which can be utilized for 3D culture without the need for FCS to enable standardization across groups worldwide (Figure 1). Finally, freezing methodology was collected from groups and recommendations made for preparing cells for cryopreservation.

2.2 | Protocol collection and invitation for study involvement

Invitations were sent to the Orthopaedic Research Society (ORS) Spine Section members and laboratories known to perform substantial NP cell culture whom were not ORS Spine Section members for inclusion within the study. Protocols were collected from 36 groups worldwide (Supplementary Material 1: Table S1) who routinely culture extracted IVD cells with an emphasis on NP cell isolation and culture using a survey (Supplementary Material 2: Data S1). Additional follow-up information was requested on inclusion of pyruvate, ascorbic acid, and cryopreservation methods. Protocols were collated and reviewed for differences and similarities in NP cell extraction protocols, composition of expansion media, and re-differentiation media. Methods were submitted for mouse, rat, rabbit, pig, cow, dog, and human IVDs; no methods were received for goat or sheep cell extraction methodology. For analysis, species were grouped as small animals (mouse, rat, and rabbit), large animals which retain the large vacuolated NC cells at young adult age (pig and non-chondrodystrophic dogs), species which lack large vacuolated NCs in mature animals (cow, and chondrodystrophic dogs), and humans.[36] Cell extraction methods were categorized based on the type, concentration, and timing of pronase and collagenase treatment, as well as the size of the cell strainer. Cell expansion and differentiation media were categorized based on the type of media, FCS concentration (or exclusion), inclusion of pyruvate, ascorbic acid (and type) and use of growth factors, oxygen (O_2) concentration, and glucose concentration. NP cell cryopreservation methods were collected and categorized for freezing media recipe, and freezing and thawing methods.

2.3 | Ethical considerations

All experimental work described in this study utilized waste animal and human tissues; specific details are included for each species below. Human IVD tissue was obtained from patients undergoing microdiscectomy surgery for the treatment of nerve root compression as a result of IVD herniation or post-mortem (PM) examination with informed consent of the patients or relatives. Ethical approval was granted from Sheffield Research Ethics Committee (09/H1308/70), or from deidentified lumbar spine segments donated by LifeShare of the Carolinas as part of a LifeShare Research Review Board-approved project agreement.

2.4 | Extraction method optimization

To develop recommendations for cell isolation methodology key differential factors were tested experimentally to identify extraction methodology which obtained highest cell yield while maintaining key phenotypic factors seen in vivo. Extraction methodologies were investigated from NP tissue of rats, rabbits, pigs, dogs, cows, and humans.

2.5 | Small animal NP extraction

2.5.1 | Rat

To determine variables for the rat NP cell isolation protocol, in addition to the submitted protocols a literature review was conducted in PubMed using the search terms "rat," "nucleus pulposus," and "culture." Initial results yielded 342 papers, of which 210 met our inclusion criteria (i.e., written in English and described the isolation of rat NP cells). The following digestion parameters were extracted from each manuscript: pre-digestion (i.e., enzyme type, enzyme concentration, duration), digestion (i.e., enzyme type, enzyme concentration, duration), pyruvate concentration, and strainer mesh size. Digestion parameters from the 210 manuscripts were grouped by corresponding author to yield 116 protocols.

A review of these methods showed that in 56% of protocols, a pre-digestion was used with the most common pre-digestion enzymes of trypsin (34.5%) and pronase (12.9%). The most commonly reported concentration of pronase was 0.2%–0.4% with a digestion time of 1 h at 37°C. This activity range was determined to be ~14 U/ml. This was similar to the pronase concentrations used in the other animal NP cell isolation protocols and was thus selected for this study. For breakdown of the collagen matrix, the most common digestion enzyme was collagenase type II (45.2%). Hence, this enzyme was chosen for these experiments. The two most common collagenase type II concentrations were 0.1%–0.125% and 0.2%–0.25% for up to 6 h. Since no consensus existed in the literature, collagenase concentration was considered a key variable. Concentrations were converted to approximate activities, which yielded approximate low and high activities of 100 and 400 U/ml, respectively. The timing of the pronase predigestion and collagenase was considered an important variable for optimizing cell isolation procedures in all species. Tissue was either digested with pronase and collagenase in combination ("combined"), or sequentially ("sequential"), with pronase pre-digestion followed by collagenase. The rationale for combined was that rat IVD tissue is

limited in mass, and increased manipulation and washing stages promotes a higher risk of cell loss. Based on protocols for other species and since most protocols in the literature did not clearly specify, pyruvate concentration was set at 110 mg/L and a 70 µm strainer mesh was employed as the most common methods reported in the survey.

The study design included the following four groups: (Digestion method 1): sequential-low (Seq-LO) collagenase (100 U/ml), (Digestion method 2): sequential-high (Seq-HI) collagenase (400 U/ml), (Digestion method 3): combined-low (Combi-LO) collagenase (100 U/ml) and (Digestion method 4): combined-high (Combi-HI) collagenase (400 U/ml). All conditions used pronase at a concentration of 14 U/ml. For the sequential conditions, tissue was pre-digested with pronase (Millipore Sigma, 53 702) for 1 h and subsequently digested with collagenase II (Gibco, 17101-015) for 5 h. For the combination conditions, the tissue was digested with both pronase and collagenase for 6 h in total.

2.5.2 | Rat samples

Three laboratories followed the study design outlined above: Trinity College Dublin, Ireland; the University of Hong Kong, China; and the Icahn School of Medicine at Mount Sinai, New York. At Trinity College Dublin, rat tails were obtained from ~5-month-old male Wistar rats (Trinity College Dublin Comparative Medicine Unit). At Mount Sinai, rat tails were obtained from 5-month-old male Sprague–Dawley rats (Charles River Laboratory, Wilmington, MA). At University of Hong Kong, rat tails were obtained from 4 to 5 months old female Sprague–Dawley rats (BesTest Bio-Tech, China). Across all three laboratories, the rats were obtained within 1 h of euthanasia. For all protocols, rat caudal spines were excised near the base of the tail at disc level C4–C5 (Figure 2A). The skin was removed, and the NP was exposed by inserting a number 11 blade near a bounding endplate along the transverse plane, avoiding the central plane of the disc in order to maintain NP integrity and avoid tissue shearing. The NP tissue was then removed using a needle-point forceps and weighed (Figure 2B). While cell extraction from individual discs is possible, the groups used a minimum of 6-discs per tail and a total of three tails per digestion condition to ensure sufficient cell number for cell culture and expansion.

2.5.3 | Digestion of rat NP tissue

Following harvesting, NP tissues were washed once with phosphate-buffered saline (PBS) with 3% v/v penicillin–streptomycin (P/S) + 1.5% v/v amphotericin B. Tissues then underwent enzymatic digestion using either the sequential or combined digestion steps described above. Solutions were prepared from high glucose Dulbecco's Modified Eagle Media (hgDMEM) with 110 mg/L pyruvate and 1% P/S and were sterile filtered before use. All digestions were performed at 37°C in 50 ml centrifuge tubes at a ratio of 1 ml of digestion solution per disc, for a period of 6 h under constant rotation (10 rpm). For the sequential digestions, tissues were first incubated in pronase for 1 h. Digestion medium was then centrifuged at 500*g* for 5 min, the supernatant aspirated, and the resulting pellets were resuspended in

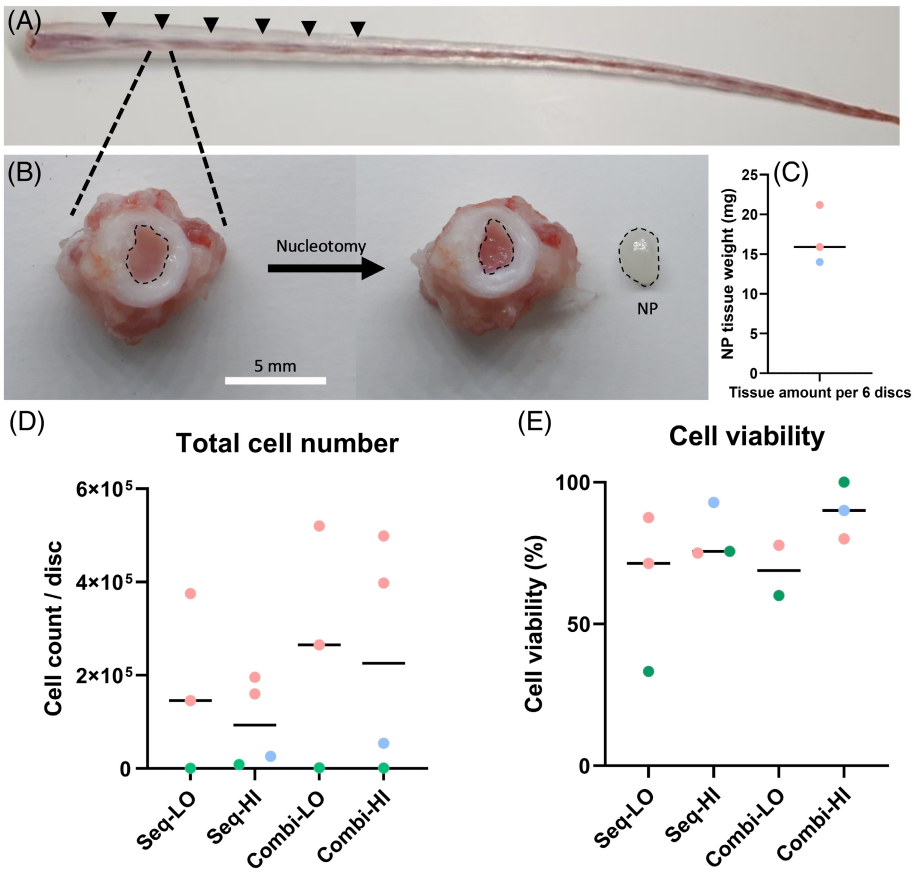


FIGURE 2 Cells were isolated from rat NP tissue using different methods: (A) intact rat tail, black arrow heads indicate individual discs. (B) Cartilage endplate (CEP) removed and NP tissue isolated for digestion. (C) NP tissue wet weights (mg) for six discs. (D) Total cell yield (×105) and (E) Cell viability (%) for each digestion protocol—sequential low/high collagenase, combined low/high collagenase. Pink dots represent Trinity College Dublin data, the blue dots are University of Hong Kong, and green dots are Icahn School of Medicine at Mount Sinai. One-way ANOVA with Tukey's post hoc tests for pairwise comparisons between extraction protocols was performed to assess significance. All $P > 0.05$.

collagenase type II solution and gently triturated with an 18G needle to break up clumps. The digests were further incubated for 5 h at 37°C. For combined digests, NP tissue was suspended in 1 ml/disc of pronase, collagenase digestion media in 50 ml tubes and incubated for 6 h at 37°C with rotation (10 rpm). NP tissue was gently triturated with an 18G needle after approximately 1–2 h to maintain consistency as per the sequential digestion protocol. Following digestion, cells were passed through a 70 µm cell strainer, centrifuged (500*g*, 5 min) and resuspended in PBS. Total cell yield and viability were determined using trypan blue. RNA was extracted and RT-qPCR analysis performed for phenotypic markers (see downstream target analysis below). Isolated rat NP cells were not utilized for further culture studies and were all utilized for immediate direct extraction analysis.

2.6 | Rabbit

2.6.1 | Rabbit samples

Rabbit spines were obtained from New Zealand White rabbits within 2 h of euthanasia, donated by collaborators from University of Pittsburgh Department of Ophthalmology (8- to 10-week-old rabbits, ~2.5 kg) or Rush University Department of Physiology & Biophysics (11- to 15-week-old rabbits, ~2.75 kg rabbits).

2.6.2 | Digestion of rabbit NP tissue

Based on the four rabbit NP cell extraction protocols submitted by different groups worldwide, multiple trials of isolating rabbit NP cells were conducted to determine which method was the most effective in isolating cells. Some of the more common reagents and conditions reported in the submitted protocols and therefore utilized in all tests include: (1) antibiotics: P/S; (2) media: hgDMEM/F12; (3) Serum concentration: 10% FCS; (4) enzymes: pronase and collagenase type P; (5) Media additions: ascorbic acid; and (6) Oxygen concentration: 21%. The four protocols varied in their enzyme digests: concentration, duration, and whether performed sequentially or in combination.

Briefly, in each trial, 6–10 IVDs were isolated and washed quickly three times in saline and 50 µg/ml gentamicin and three times for 10 min each in saline or DMEM/F12 containing 50 µg/ml gentamicin. NP tissues from 6–10 IVDs for each digestion protocol were isolated in sterile conditions and underwent enzymatic digestion using four different protocols based on those submitted to the original questionnaire. The collected NP tissue underwent enzymatic digestion in hgDMEM/F12: (Digestion Method 1): 0.05% (30.84 U/ml) pronase (MilliporeSigma, 53702) for 45 min and 0.01% (0.16 U/ml) collagenase P (Roche, MilliporeSigma, 11213873001) for 1 h, sequentially. (Digestion Method 2): 0.2% (123.35 U/ml) pronase for 1 h and 0.01% (0.16 U/ml) collagenase P overnight, sequentially. (Digestion Method 3): 0.1% (61.68 U/ml) pronase combined with 0.025% (0.4 U/ml) collagenase P for 2 h, or (Digestion Method 4): 0.4% (246.71 U/ml) pronase combined with 0.0125% (0.2 U/ml) collagenase for 1.5 h, in ~2.5 ml per disc.

NP cells were washed three times using hgDMEM/F12 (10-090-CV, Corning) supplemented with 5% FCS (FB-01, Omega) and centrifugation at 453*g* for 10 min at 4°C, and passed through a 70 µm strainer (Falcon, Corning, 352350). NP cells were resuspended and cultured in complete media, DMEM/F12 containing 10% FCS, 2 mM L-glutamine, 25 µg/ml L-Ascorbic Acid (L-AA), and 1% P/Sat 21% O_2, 5% CO_2. Following extraction, live cell number per disc was calculated, and used for RNA extraction and real-time PCR analysis performed for phenotypic markers (see downstream analysis below).

2.7 | Large animal NP extraction

2.7.1 | Pig

Pig samples

Pig spines were obtained from 4-month-old animals through the Washington University Division of Comparative Medicine within 6 h of animal sacrifice. Lumbar IVDs ($n = 3$ total) were excised under sterile conditions and NP tissue was collected by precise separation from the AF and CEPs. NP tissue was isolated, finely chopped, and prepared for enzymatic cell isolation.

Digestion of pig NP tissue

NP tissue was weighed prior to digestion to enable calculation of cell yield per gram of tissue. The collected NP tissue underwent enzymatic digestion using two different protocols and different timepoints of extraction based on the submitted protocols. Solutions were prepared from hgDMEM with collagenase type II (0.5 mg/ml [16 U/ml] or 2 mg/ml [64 U/ml], Sigma C6885) both without (C only) or in combination with (C + P) the addition of pronase (2 mg/ml [14 U/ml], Roche 11459643001). In addition, 4 h of digestion (with 0.5 mg/ml collagenase above) was compared with 16 h digestion solution of hgDMEM with collagenase type II (0.5 mg/ml [16 U/ml]), both without or in combination with pronase (2 mg/ml [14 U/ml] for 4 h, 0.4 mg/ml [3 U/ml] for 16 h). Solutions were supplemented with heat-inactivated 5% FCS (Cat: 10438-026, Thermo Fisher Scientific, Waltham, MA) and tissues were incubated with the digestion medium at 30 ml per g of tissue for 4 or 16 h in the respective digestion media as described. Following digestion, cells were passed through a 70 µm cell strainer and centrifuged at 300*g*. Cell yield and viability were determined via automated cell counting (Countess® AMQAX1000, Thermo Fisher Scientific). Fifty thousand cells per sample ($n = 3$ samples per donor) were used for direct RNA extraction and real-time PCR analysis (see below).

2.8 | Dog NP cell extraction

2.8.1 | Dog samples

Complete dog spines were collected from three female chondrodystrophic beagle dogs (3–4 years of age) that had been euthanized in unrelated research studies (approved by the Utrecht University Animal Ethics

Committee, DEC study number 2016.II.529.002). Beagle dogs develop spontaneous IDD at about 1 year of age and are considered a good animal model to study human IDD.[37] Beagle dogs have ~100% NP cells at the age studied, and (almost) no NCs.[38] IVDs (scored as Thompson grade II/III) from all donors were opened under sterile conditions and NP tissue was collected by precise separation from the AF and CEPs.[37,38]

2.8.2 | Digestion of dog NP tissue

NP tissue was weighed prior to digestion to enable calculation of cell yield per g of tissue. The collected NP tissue underwent enzymatic digestion in hgDMEM + Glutamax (31 966, Invitrogen): Digestion method 1:0.5 mg/ml (62.5 U/ml) collagenase type II (LS004177, Worthington) for 4 h; Digestion method 2:0.75 mg/ml (5.25 U/ml) pronase (10165921001, Roche Diagnostics) for 30 min followed by 0.05 mg/ml collagenase type II for 4 h; Digestion method 3: 0.5 mg/ml collagenase type II for 16 h; Digestion method 4: 0.75 mg/ml pronase (30 min) followed by 0.05 mg/ml collagenase type II for 16 h, with a minimum 20 ml/g tissue. NP cells were passed through a 70 µm strainer, washed three times using hgDMEM + Glutamax supplemented with 10% heat inactivated FCS (Gibco, 10500064) and 1% P/S (P11-010, GE Healthcare Life Sciences), and centrifuged at 500*g* for 5 min. Cell numbers and viability were determined using a TC20 automated cell counter (145-0101, Bio-Rad). One hundred thousand cells ($n = 3$ per donor and condition) were used for RNA extraction and RT-qPCR to assess cell growth, anabolism/catabolism, apoptosis, and phenotypic markers (see below).

2.9 | Cow NP cell extraction

2.9.1 | Cow samples

Cow tails from 9 to 18 month old cows were obtained from a local abattoir (Sheffield UK; $n = 3$). The tails were rinsed with Lifoscrub and tap water to remove dirt and hair. The whole tail was immersed in 10% betadine solution for 10 min. Briefly, tails were dried with sterile gauze and placed under the sterile hood. Using a scalpel blade number 20 as much tissue around the spine as possible was removed to ensure easy localization of the IVD for dissection. Caudal IVDs were excised, and NP tissue was isolated from the surrounding AF and CEP tissue, finely chopped, and extraction of NP cells was optimized using varying types and concentrations of collagenase based on the submitted protocols. While pronase was utilized in a number of the submitted protocols, prior evidence suggests that pronase pre-treatment is not required in cow tissues,[39] and thus was excluded from investigation in this study.

2.9.2 | Digestion of cow NP tissue

NP tissue was weighed prior to digestion to enable calculation of cell yield per g of tissue. Collagenase type I (Gibco 17018029) and type II (Gibco 17101015) were tested at either 64 or 256 U/ml for a digestion time of either 4 or 16 h. Enzyme digests were completed in hgDMEM containing 1% P/S, and 50 µg/ml amphotericin B with 20 ml/g tissue digestion solution under agitation at 37°C. Following digestion, cells were passed through a 70 µm cell strainer, counted, and used for direct RNA extraction (1×10^5 cells) and cell culture to assess cell growth and phenotypic markers.

2.10 | Human NP cell extraction

2.10.1 | Human samples

Human IVD tissue was obtained from patients undergoing microdiscectomy surgery for the treatment of nerve root compression as a result of IVD herniation with informed consent of the patients or relatives (Sheffield Research Ethics Committee [09/H1308/70]). This study utilized 10 surgical samples from 10 individuals (Table S1), which were graded histologically for signs of histological degeneration as reported recently[21]; as some tissue samples only contained NP tissue histological grading was performed for NP tissue only and reported on a scale of 0–9,[21] where 0–3 is considered non-degenerate, 4–6 moderate degeneration, and 7–9 severe degeneration.

2.10.2 | Digestion of human NP tissue

Based on survey data collected, and due to low volume of human tissue available from surgical samples, initial optimization using cow NP cell extraction was utilized to inform human optimization. Human NP cell extraction utilized collagenase type II at 64 U/ml (0.5 mg/ml) which was selected from cow testing for 4 or 16 h. Prior to digestion, human NP tissue was washed twice with PBS containing 1% v/v P/S (Life Technologies, Paisley, UK). IVD was morphologically separated into NP and AF tissue. NP tissue was weighed prior to digestion to enable calculation of cell yield per g of tissue. Human NP cells were isolated using collagenase type II (Sigma Aldrich, Gillingham, UK, 17101-015) at a concentration of 64 U/ml in hgDMEM containing 1% v/v P/S at 20 ml/g tissue for either 4 or 16 h on 600 rpm orbital shaker at 37°C. Following digestion, NP cells were passed through a 70 µm cell strainer, counted using trypan blue exclusion and hemocytometer count, and used for direct RNA extraction and cell culture. Following extraction, human NP cells (patient samples $n = 7$, age = 42 ± 9; Table 1) were expanded in monolayer in hgDMEM (Life Technologies, Paisley, UK) supplemented with 10% v/v heat-inactivated FCS (Life Technologies, Paisley, UK), 1% P/S, 2 mM glutamine, 50 µg/ml amphotericin B, and 50 µg/ml L-AA (Base media) and maintained at 37°C in a humidified atmosphere containing 21% O_2, 5% CO_2 to determine influence of extraction method on cell expansion and phenotype.

2.11 | Cell expansion optimization

Culture of NP cells was investigated to determine the effect of basal media (within rabbit, cows, and humans), alternatives to fetal calf

TABLE 1 Human IVD tissue used in this study (Sheffield)

Donor	Disc level	Sex	Age	Histological grade of NP tissue (0–9)	Classification
HD563	L3/4	M	44	5	Moderately degenerate
HD564	L5/S1	M	41	4	Moderately degenerate
HD565	L5/S1	M	31	3	Non-degenerate
HD569	L5/S1	M	53	6	Moderately degenerate
HD574	L5/S1	F	42	8	Severely degenerate
HD575	L5/S1	M	32	5	Moderately degenerate
HD576	L5/S1	M	49	6	Moderately degenerate
HD577	L3/4	F	72	6	Moderately degenerate
HD580	L4/5	N/A	N/A	5	Moderately degenerate
HD585	L4/5	M	34	5	Moderately degenerate

serum (FCS) (within dogs and humans) and differential substrates (within dog, pigs, and human). Selected species were identified here to represent key species utilized for such studies as initial indications for recommendations.

2.12 | Basal media optimization

2.12.1 | Rabbit

To test for phenotypic changes due to media differences, directly extracted NP cells were plated in monolayer and cultured in complete hgDMEM/F12 until passage 1 (average of 8 days) and cultured in complete test media (hgDMEM/F12, hgF12, or hgDMEM) at 21% O_2, 5% CO_2, and 37°C until passage 2 (average of 7 days) before collection.

2.12.2 | Cow

To test for phenotypic changes and growth rate differences, directly extracted cow NP cells were cultured in complete hgDMEM (Gibco 10569010) or hgDMEM/F12 (Gibco 10565018) containing 10% FCS (Gibco FCS qualified HI 10500064), P/S (Gibco 15070063), 2 mM glutamine (Gibco 25030024), 50 µg/ml amphotericin B (Sigma A2942-100ML) and 50 µg/ml L-AA (Sigma A5960-100G) at 21% O_2, 5% CO_2, and 37°C. Cells were cultured up to passage 3, taking cells for RNA isolation at each passage. Cumulative population doubling (cPD)[40] was determined during culture within hgDMEM or hgDMEM/F12 complete media. Following expansion in monolayer for 2 passages cells were transferred to alginate bead culture and cultured for 2 weeks to investigate re-differentiation capability.

Alginate bead culture

Alginate bead culture was established to induce re-differentiation of dog, cow, and human NP (Sheffield, UK) cells following expansion in culture; alginate is the most commonly utilized 3D culture system for redifferentiation.[12] NP cells expanded in monolayer culture to passage 2 at 21% O_2, 5% CO_2, and 37°C were resuspended in sterile-filtered 1.2% (w/v) alginic acid (A2033, Sigma-Aldrich) in 0.15 M NaCl at a density of 4×10^6 cells/ml.[41–43] The resulting cell-alginate suspension was polymerized by passing through a 20G needle drop-by-drop into 0.2 M $CaCl_2$ to produce alginate beads and left for 10 min to fully gel at 37°C. Newly formed beads were washed twice with 0.15 M NaCl to remove excess $CaCl_2$ and washed twice with hgDMEM before complete culture media was added. Alginate beads were polymerized and cultured in 24-well plates (Thermo Fisher Scientific, 142475) with 6 beads per well and cultured as described below.

2.13 | Human

To assess phenotypic changes due to basal media and culture surface coating differences (laminin vs. uncoated) in monolayer culture. Human NPs were extracted from deidentified IVD tissue from four individuals (18 y.o. M, 38 y.o. M, 41 y.o. M, and 68 y.o. M) received from LifeShare of the Carolinas as part of a LifeShare Research Review Board-approved project agreement and digested with 780 U/ml collagenase type II (Worthington Biochemical, LS004177) and 14 U/ml pronase (Sigma-Aldrich, 11459643001) in hgDMEM (Cytiva, SH30243) containing 150 µg/ml gentamycin (Thermo Fisher Scientific, 15750060), 100 µg/ml kanamycin (Sigma-Aldrich, K0129), and 1 µg/ml amphotericin B (Thermo Fisher Scientific, 15290018) for 4 h at 37°C with agitation (note: extraction was performed prior to optimization of recommended methodology so current local lab method applied). Following digestion, NP cells were passed through a 70 µm cell strainer, pelleted by centrifugation (200*g*, 10 min), and seeded at 20 000–40 000 cells per cm^2 in 6-well plates on either uncoated tissue culture plastic or on plastic coated with 2 µg/ml or 20 µg/ml laminin (Trevigen, 3446-005-01). Cells were cultured in either hgDMEM (Cytiva, SH30243) or Ham's F12 Nutrient Mix (Thermo Fisher Scientific, 11765062), both supplemented with 1% (100 U/ml) P/S (Thermo Fisher Scientific, 15140122) and 10% FCS (Sigma-Aldrich, F2442) at 21% O_2, 5% CO_2, and 37°C. At confluency, cells were collected for qRT-PCR gene expression analysis.

TABLE 2 qRT-PCR assays used for quantitative PCR

Gene	Rat	Rabbit	Pig	Dog	Cow	Human (Charlotte, NC)	Human (Sheffield, UK)
Housekeeping genes							
18S		Hs99999901_s1	Hs99999901_s1		Hs99999901_s1		Hs99999901_s1
B2M			Ss03391154_m1				
GAPDH	Rn01775763_g1			Primers: 5′-TGTCCCCACCCCCAATGTATC-3′ and 5′-CTCCGATGCCTGCTTCACTACCTT-3′; Amplicon: 100, Annealing temp: 58, Accession: NM_001003142	Hs99999905-m1	Hs02758991_g1	Hs99999905-m1
HPRT				Primers: 5′-TATTGTAATGACCAGTCAACAG-3′ and 5′-GGTCCTTTTCACCAGCAAG-3′; Amplicon: 192, Annealing temp: 60, Accession: NM_000194.2			
RNA45S5						Hs05057753_g1	
RPS19				Primers: 5′-CCTTCCTCAAAAAGTCTGGG-3′ and 5′-GTTCTCATCGTAGGGAGCAAG-3′; Amplicon: 95, Annealing temp: 61, Accession: XM_005616513			
Phenotypic markers							
ACAN	Rn00573424_m1	Oc06726465_m1	Ss03374823_m1	Primers: 5′-GGACACTCCTTGCAATTTGAG-3′ and 5′-GTCATTCCACTCTCCCTTCTC-3′; Amplicon: 111, Annealing temp: 62, Accession: NM_000088.3	Hs00153936-m1	Hs00153936_m1	Hs00153936-m1
T	Rn01527349_m1	Oc03395780_m1	Ss03374654_g1				
CAR12		Oc03397162_m1					
CCND1				Primers: 5′-GCCTCGAAGATGAAGGAGAC-3′ and 5′-CAGTTTGTTCACCAGGAGCA-3′; Amplicon: 117, Annealing temp: 60, Accession: NM_001005757.1			
FOXF1					Hs00230962-m1		Hs00230962-m1
HIF1A		Oc03398626_m1					
KRT18	Rn01533360_g1	Primers: 5′-ACT ACT TCA AGG CCA TCG-3′ and 5′-AAG TCA TCA GCA GCA AGA-3′; Probe: 5′-/56-FAM/TT TGC CAA C/Zen/T CCG TGG ACA ACG C/3IABkFQ/-3′	Ss03377383_m1			Hs02827483_g1	

(Continues)

TABLE 2 (Continued)

Gene	Rat	Rabbit	Pig	Dog	Cow	Human (Charlotte, NC)	Human (Sheffield, UK)
KRT19		Oc06701460_m1		Primers: 5′-GCCCAGCTGAGCGATGTGC-3′ and 5′-TGCTCCAGCCGTGACTTGA TGT-3′; Amplicon: 86, Annealing temp: 64, Accession: NM_001253742			
KRT8				Primers: 5′-CCTTAGGCGGGTCTCTCGTA G-3′ and 5′-GGGAAGCTGGTGTCTGAGTC-3′; Amplicon: 149, Annealing temp: 63, Accession: XM_543639	Hs02827483-g1		Hs02827483-g1
PAX1					Hs00196352-m1		Hs00196352-m1
SHH	Rn00568129_m1	Oc06796169_m1	Ss04327682_m1				
COL1A1	Rn01463848_m1	Oc03396113_m1	Ss03373340_m1	Primers: 5′-TCCAACGAGATCGAGATCC-3′ and 5′-AAGCCGAATTCCTGGTCT-3′; Amplicon: 151, Annealing temp: 61, Accession: XM_005616513	Hs00164004-m1	Hs00164004_m1	Hs00164004-m1
COL2A1	Rn01637087_m1	Oc03396134_m1	Ss03373344_g1	Primers: 5′-GCAGCAAGAGCAAGGAC-3′ and 5′-TTCTGAGAGCCCTCGGT-3′; Amplicon: 151, Annealing temp: 62, Accession: XM_005636674	Hs00156568-m1	Hs00264051_m1	Hs00156568-m1
Apoptotic markers							
BAX				Primers: 5′-CCTTTTGCTTCAGGGTTTCA-3′ and 5′-CTCAGCTTCTTGGTGGATGC-3′; Amplicon: 108, Annealing temp: 58, Accession: NM_001003011.1			
BCL2				Primers: 5′-ATCGCCCTGTGGATGACTGAG-3′ and 5′-CAGCCAGGAGAAATCAAACAGA GG-3′; Amplicon: 125, Annealing temp: 64, Accession: XM_005616513			

Note: All pre-designed Taq Man Primers from Life Technologies, PrimeTime® Mini qPCR Assay Rabbit Keratin 18 (Integrated DNA Technologies, Coralville, IA). Dog SYBR green primers designed using Perlprimer, Bax from (doi: 10.1556/APhysiol.100.2013.4.8).

2.14 | Effect of passage number on NP phenotype

2.14.1 | Rabbit

To test for phenotypic changes in passage number, passage 0 (P0) cells were plated directly after isolation, and cultured in complete media, DMEM/F12 containing 10% FCS, 2 mM L-glutamine, 25 µg/ml ascorbic acid, and 1% v/v P/S at 21% O_2, 5% CO_2. These cells were collected after reaching 70% of confluency at ~9 days. These cells were passaged two times (detached from plates with trypsin, cultured until 70% confluency) at 21% O_2, 5% CO_2, and 37°C and collected as passages 1 and 2 (P1 and P2), ~10 days per passage.

2.14.2 | Pig

To test for phenotypic changes in passage number cells were plated directly after isolation upon gelatin- or Geltrex™-coated flasks and collected after reaching 70% of confluency. To prepare coated flasks, tissue culture flasks were incubated overnight at 37°C with 0.1% w/v gelatin (Sigma, G1393) or 0.1% w/v Geltrex™ (ready-to-use, Thermo Fisher A1569601) at 21% O_2, 5% CO_2, and 37°C. These cells were passaged two times (detached from plates with 0.05% trypsin/EDTA Thermo Fisher, 25-300-062) each time after culture until 70% confluency and collected as passages 2 and 3 (P2 and P3), ~10 days per passage. At the end of each cell collection, RT-PCR was performed on mRNA collected from 50 000 cells per sample ($n = 3$ replicates per donor) to evaluate mRNA levels for a subset of NP-cell specific markers (Table 2).

2.14.3 | Human

To test for phenotypic changes in human NP cells (Charlotte, NC) related to passage number in monolayer culture, deidentified IVD tissue from four individuals (18 y.o. M, 38 y.o. M, 41 y.o. M, and 68 y.o. M) was received from LifeShare of the Carolinas as part of a LifeShare Research Review Board-approved project agreement and digested as described above in basal media optimization and seeded at 20 000–40 000 cells per cm^2 in 6-well plates on uncoated tissue culture plastic and cultured in hgDMEM (Cytiva, SH30243) containing 1% (100 U/ml) P/S (Thermo Fisher Scientific, 15140122) and 10% FCS (Sigma-Aldrich, F2442) at 21% O_2, 5% CO_2, and 37°C. After reaching 80% confluency, cells were passaged with 0.05% Trypsin–EDTA (Sigma-Aldrich, 59417C). Some cells from each passage were re-seeded up to passage 4, while the remaining cells were collected for qRT-PCR gene expression analysis.

2.14.4 | Alternatives to FCS in expansion media

Due to the high batch-to-batch variability in FCS, we tested alternatives which have been reported in the disc community for 3D culture methods to test whether these systems could also be deployed for 2D culture expansion, or culture media containing lower FCS concentrations.

2.14.5 | Dog

NP cells obtained using the optimal digestion methods (highest viability and cell numbers): sequential digestion with pronase for 30 min and collagenase for 4 or 16 h were utilized to test basal media for cell culture expansion. Three different expansion media were tested based on those reported in submitted protocols: (Expansion medium 1—10% FCS): hgDMEM + Glutamax with 10% FCS, 0.1 mM Ascorbic acid 2-phosphate (Asap; A8960, Sigma-Aldrich) and 0.5% Fungizone (15290-018, Invitrogen); (Expansion medium 2—5% FCS + FGF): hgDMEM + Glutamax with 5% FCS, 1 ng/ml FGF-2 (PHP105, AbD-Serotec), 0.1 mM Asap, 0.5% Fungizone, 1% ITS + premix (354 352, Corning Life Sciences), 0.04 mg/ml L-proline (P5607, Sigma-Aldrich), and 1.25 mg/ml Human Serum Albumin (HSA, Sanquin Research); (Expansion medium 3—5% FCS); hgDMEM + Glutamax with 5% FCS, 0.1 mM Asap, 0.5% Fungizone, 1% ITS+ premix, 0.04 mg/ml L-proline, and 1.25 mg/ml HSA. All cells were expanded at ~5000 cells/cm^2 at 5% O_2, 5% CO_2, and 37°C. Expansion medium was changed twice weekly. The digested cells cultured in all three expansion conditions (10%, 5% + FGF, 5%) were used to determine the cumulative population doubling (cPD)[40] from passage 0 to 3. Finally, 100 000 cells ($n = 3$ replicates per donor and expansion condition) were used for RNA isolation and RT-qPCR (see below) to determine NP cell gene expression levels.

2.14.6 | Dog NP cell redifferentiation

After Dog NP cells (expanded in all three conditions) reached 80%–90% confluence in P2, they were suspended in alginate as described above. Empty (no cell-containing) and NP cell-containing beads were cultured for 14 days at 5% O_2, 5% CO_2, 37°C, in two different redifferentiation media: (Redifferentiation medium 1: FCS): hgDMEM + Glutamax with 10% FCS, 0.1 mM Asap, 0.5% Fungizone; and (Redifferentiation medium 2: no FCS): hgDMEM + Glutamax with 0.1 mM Asap, 0.5% Fungizone, 1% ITS + premix, 0.04 mg/ml L-proline, and 1.25 mg/ml HSA. In total, six different conditions were tested (three different expansion (E) media and two different redifferentiation (R) media: (1) $E_{10\%}$ R_{FCS}, (2) $E_{10\%}$ R_{noFCS}, (3) $E_{5\%+FGF}$ R_{FCS}, (4) $E_{5\%+FGF}$ R_{noFCS}, (5) $E_{5\%}$ R_{FCS}, and (6) $E_{5\%}$ R_{noFCS}). Six beads were cultured per well in a 24-wells plate (CLS3473-24EA, Corning) in 700 µl redifferentiation medium. After 0 and 14 days, six beads were pooled ($n = 3$ replicates per donor and condition) for RNA isolation and RT-qPCR, see below. Also, three beads were pooled ($n = 2$ replicates per donor and condition) for glycosaminoglycan (GAG) and DNA measurements,[43] while three beads ($n = 2$ replicates per donor and condition) were pooled for histological purposes.

2.14.7 | Laminin coating

Furthermore, to test whether dog NPCs were able to expand without FCS, they were expanded in hgDMEM + Glutamax, 1 ng/ml FGF-2, 0.1 mM Asap, 0.5% Fungizone, 1% ITS + premix, 0.04 mg/ml L-proline, and 1.25 mg/ml HSA with T175 flasks that were coated with 5 µg/ml human rLaminin-521 (Corning, 354 221).

2.14.8 | Human

To determine the effects of media supplementation with FCS versus serum-free alternatives as well as differences in culture surface coating (Geltrex vs. uncoated) in monolayer culture, human NP cells (Charlotte, NC) were isolated as described in the basal media optimization section above from deidentified IVD tissue and seeded at 20 000–40 000 cells per cm^2 in 6-well plates wells on either uncoated tissue culture plastic or plastic coated with 0.1 mg/ml Geltrex (Thermo Fisher Scientific, A1413201) according to manufacturer's protocol. Cells were cultured with one of three media formulations, all of which contained hgDMEM (Cytiva, SH30243) and 1% (100 U/ml) P/S (Thermo Fisher Scientific, 15140122) in addition to one of three supplement combinations: (1) 10% FCS (Sigma-Aldrich, F2442); (2) 1.25 mg/ml Albumax I (Thermo Scientific, 11020021), 40 µg/ml proline (Sigma-Aldrich, P5607), and 1% ITS-X (Thermo Scientific, 51500056); or (3) 1.25 mg/ml Albumax, 40 µg/ml proline, 1% ITS-X, and 1 ng/ml FGF-2 (Bio-Rad, PHP105) at 5% O_2, 5% CO_2, and 37°C. At confluency, cells were collected for qRT-PCR gene expression analysis.

2.14.9 | Fibronectin-coated flasks for the culture of NP cells without FCS

Human NP cells from three patients in Sheffield, UK (HD563, HD564, HD565, Table 1) were pooled and cultured in hgDMEM containing P/S, 2 mM glutamine, 50 µg/ml amphotericin B and 50 µg/ml L-AA with either (1) 10% FCS; (2) 1% ITS-X; (3) 1% ITS-X and 1 ng/ml FGF-2; (4) 1.25 mg/ml Albumax, 1% ITS-X and 1 ng/ml FGF-2; (5) 1.25 mg/ml Albumax and 1% ITS-X; (6) 1.25 mg/ml Albumax, 1% ITS-X, 40 µg/ml L-proline; (7) 1.25 mg/ml Albumax, 1% ITS-X and 40 µg/ml L-proline and 1 ng/ml FGF-2 at 21% O_2, 5% CO_2, and 37°C. To assist the attachment of NP cells in culture without FCS in the medium, wells of a 6-well plate were coated in 500 µl of 2 µg/ml fibronectin (F1141, Sigma) as per manufacturers' instructions. Once the entire area was covered, the excess volume was removed and the flasks were left to air dry for at least 45 min before introducing the cells and the medium.

2.14.10 | Alternatives to FCS during 3D differentiation

After resuspending human NP cells (from three patients: HD569, HD577, HD580, Sheffield, UK) (Table 1) into alginate beads, cells were re-differentiated during culture for 2 weeks in 3D alginate culture at 37°C, 5% O_2, and 5% CO_2. During the 2-week re-differentiation period alginate beads ($n = 24$ per patient) were cultured in one of three different media to test the effect of FCS and alternatives on the re-differentiation process. The basal composition of each media treatment was identical (low glucose DMEM, 2 mM L-glutamine, 1% v/v P/S [Gibco], 25 µg/ml amphotericin B, 50 µg/ml ascorbic acid [Sigma]), with the addition of (re-differentiation media 1) 10% (w/v) FCS, (re-differentiation media 2) 1% (w/v) ITS-X, or (re-differentiation media 3) 1% (w/v) ITS-X, 40 µg/ml L-proline. Human NP cells extracted from three individual patients were used in each separate experiment.

2.15 | Downstream analysis

2.15.1 | Viability and cell count

Following extraction cell counts were calculated per disc (rat and rabbit) or per gram of tissue (pig, dog, cow, human). Viability was assessed to determine live cell numbers using trypan blue assay (rat, rabbit, pig, cow, human) or nucleocounter viability assay (dog).

2.15.2 | RNA extraction and gene expression analysis

RNA was isolated from directly extracted cells, cells cultured in monolayer, and encapsulated in alginate, methods were slightly different based on species, note harmonization of down-stream analysis was outside the scope of this manuscript which focuses on harmonization of culture methodology, thus standard methodology from each group was utilized and described separately.

2.15.3 | Rat

Cells were lysed using Trizol (Life Technologies), and RNA was subsequently isolated using a Qiagen RNeasy mini kit (Qiagen). cDNA preparation was performed using an Applied Biosystems High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), and RT-qPCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7500 Fast Real-Time PCR system data was analyzed using $2^{-\Delta Ct}$ method (Table 2).

2.15.4 | Rabbit

Total RNA was isolated from rabbit NP cells using the RNeasy Mini Kit (Qiagen Inc, Valencia, CA) with DNase digestion according to the manufacturer's instructions. RNA from each sample was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) with

random primers using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Gene expression was detected using Taqman assays specific for rabbit genes (Table 2). Amplification reactions were performed using TaqMan® Gene Expression Master Mix (Life Technologies) and 7300 Real-Time PCR System (Life Technologies). The results were calculated in reference to internal control, Eukaryotic 18 S rRNA, using $2^{-\Delta Ct}$ method on Data Assist Software (Life Technologies).

2.15.5 | Pig

For RT-PCR, cells (around 1×10^6) were lysed in TRIzol reagent (Life Technologies) and mRNA extraction was performed using the RNeasy mini kit plus DNase I digestion (Qiagen). mRNA integrity and concentration were checked on the ND-1000 Spectrophotometer (NanoDrop). mRNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Biorad). cDNA samples were diluted to a final concentration of 10 ng/µl using RNAse-free DNAse-free water. qRT-PCR was performed with pig-specific Taqman primer probes (Life Technologies) of NP-cell specific and NP-matrix related gene-specific primers (Table 2). The housekeeping gene, *18S* and *B2M* were used as internal controls. qRT-PCR reactions were performed on the StepOnePlus real-time PCR system (Applied Biosystems) in duplicate in 96-well plates in a final volume of 25 µl using standard conditions. PCR reactions contained 12.5 µl 2× universal master mix (Applied Biosystems), 1.25 µl Taqman primer probes, 9.25 µl ddH_2O, and 2 µl 10 ng/µl cDNA. Fold-differences were calculated based on measurements of $2^{-\Delta Ct}$ method ($2^{-\Delta Ct}$), where the Δ accounted for fold change over the mean of both housekeeping genes.

2.15.6 | Dog

The alginate beads were treated with sodium citrate digestion buffer (55 mM sodium citrate, Sigma-Aldrich; 0.15 M NaCl, Merck; 25 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, Sigma-Aldrich) for 5 min to remove interfering alginate.[43] RNA from all digestion, expansion, and redifferentiation samples was extracted using the RNeasy® Micro kit (74 004, Qiagen) according to the manufacturer's instructions with an additional DNase (79 254, Qiagen) step. cDNA was synthesized using the iScriptTM cDNA Synthesis Kit (170-8891, Bio-Rad) according to the manufacturer's instructions. Primer sequences were designed using PerlPrimer (http://perlprimer.sourceforge.net) or obtained from previous work (Table 2).[44,45] The three most stably expressed reference genes were chosen to normalize gene expression of the target genes. RT-qPCR was performed using the iQTTM SYBR Green Supermix Kit (Bio-Rad) and the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad).[44] Fold-differences were calculated based on measurements of $2^{-\Delta Ct}$ method ($2^{-\Delta Ct}$), where the Δ accounted for fold change over the mean of both housekeeping genes.

2.15.7 | Cow and human (Sheffield, UK)

Directly extracted and monolayer-cultured NP cells, following media aspiration and PBS washing, were lysed in Trizol (Life Technologies; $\sim 1 \times 10^6$ cells/ml Trizol). Chloroform was added to separate RNA, DNA, and protein layers. The RNA layer was isolated and molecular-grade isopropanol was added to precipitate RNA, which was then washed with molecular-grade ethanol. RNA was finally resuspended in 14 µl RNase-free water. Following 2 weeks culture, alginate beads containing human NP cells were added to 500µl alginate dissolving buffer (55 mM sodium citrate, 30 mM EDTA, 0.15 M NaCl in H_2O) (6 alginate beads/sample) for 30 min on an orbital shaker at 37°C. Samples were centrifuged at 300*g* for 10 min. Supernatant was discarded and pellets resuspended in media containing 0.4 mg/ml collagenase (Sigma) for 30 min on an orbital shaker at 37°C to degrade ECM deposited by NP cells during alginate bead culture. Samples were centrifuged for a further 10 min at 300*g*, supernatant removed and 1 ml Trizol (Life Technologies) added to each sample. RNA was extracted using RNeasy mini kit (Qiagen) following the manufacturers' guidelines. RNA was eluted from RNeasy mini kit columns with 14 µl RNase free water.

Following extraction, RNA was denatured at 60°C for 5 min and then was reverse transcribed into cDNA by adding 36 µl reverse transcriptase master mix (100U BioScript™ reverse transcriptase, 13.9% [v/v] BioScript™ 5× reaction buffer, 1.7 mM dNTPs [Bioline], 1.4 µM random hexamers [Thermo] in RNase-free water) per 14 µl RNA sample. Samples were incubated for 2 h at 42°C to synthesize cDNA, followed by 10 min at 80°C to denature the BioScript™ reverse transcriptase enzyme (Bioline) and stored at 4°C prior to RT-PCR analysis. qRT-PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) to investigate gene expression (Table 2). Reactions were prepared in duplicate using TaqMan Universal PCR Master Mix (Applied Biosystems, UK) and predesigned Primers (Applied Biosystems, UK) to a total volume of 10 µl, containing at least 10 ng cDNA. Data were analyzed according to the $2^{-\Delta Ct}$ method, with expression normalized to the mean of *GAPDH* and *18S* reference genes.

2.15.8 | Human (Charlotte, NC)

Cell lysis and RNA extraction were performed with TRI Reagent (Sigma-Aldrich, T9424), genomic DNA was removed with DNase I (Thermo Scientific, 18068015), and cDNA synthesis from total RNA was performed with Invitrogen Superscript IV First Strand Synthesis System (Thermo Scientific, 18091050) according to manufacturers' protocols. qRT-PCR was performed on a QuantStudio 3 System (Applied Biosystems) using TaqMan Fast Advanced Master Mix (Thermo Scientific, 4444963) and TaqMan Gene Expression assays (Table 2) results were normalized to the mean of the reference genes *GAPDH* and *RNA45S5* using the $2^{-\Delta Ct}$ method.

2.16 | Biochemical analysis

2.16.1 | Dog

2.16.2 | GAG and DNA measurements

To determine GAG and DNA content, the alginate beads were first solubilized by incubation in dissolving buffer (55 mM sodium citrate, 30 mM Na_2EDTA, and 0.15 M NaCl pH 6.8) for 20 min at 4°C to separate the cell and alginate fraction. Thereafter, both fractions were digested in papain digestion solution (pH 6, 200 mM $H_2NaPO_4 \cdot 2H_2O$ [21254, Boom B.V., Meppel, the Netherlands]), 10 mM EDTA (100944, Merck Millipore, Amsterdam, the Netherlands), 10 mM cysteine HCl (C7880, Sigma-Aldrich, St Louis), and 10 mM papain (P3125, Sigma-Aldrich, St Louis) overnight at 60°C. For quantification of the GAG content the dimethyl methylene blue (DMMB) assay was used. After the addition of the DMMB (pH 1.5) (341088, Sigma-Aldrich, St Louis), the absorbance was measured at 525/595 nm. To determine the GAG content of the matrix within the beads, the alginate fraction was corrected for the background signal of the alginate determined by measuring empty alginate beads. The DNA content in both fractions was measured using the Qubit® dsDNA High Sensitivity Assay Kit (Q32851, Invitrogen, Paisley, UK).

2.17 | Immunohistochemistry and histology analysis

2.17.1 | Dog

At day 14 of redifferentiation, the alginate beads of all three donors and six conditions were fixed overnight in 100 mM $CaCl_2$ in 4% neutral buffered formaldehyde solution and thereafter dehydrated in ethanol.[43] The next day, the alginate beads were embedded in paraffin, 5 µm sections were cut and stained with H&E (109249 and 115935, Merck). Immunohistochemistry (IHC) staining for collagen type I and II was performed as described previously.[44] IHC for aggrecan (mouse monoclonal recombinant antibody, 4 µg/ml; ab3778, Abcam) was performed for collagen type I and II, but with 60 instead of 30 min of 1 mg/ml pronase and 10 mg/ml hyaluronidase antigen retrieval treatment. Cytokeratin 19 (mouse monoclonal antibody, 10 µg/ml; ab9221, Abcam) IHC was performed without any antigen retrieval. Cytokeratin 8 (mouse monoclonal antibody, 10 µg/ml; MA1-19037, Thermofisher) and PAX1 (rabbit polyclonal, 4 µg/ml; ab203065, Abcam) IHC was performed with 10 mM citrate buffer (pH 6, 70°C) antigen retrieval for 60 and 30 min, respectively. As negative control in all IHCs, normal mouse IgG_1 (X0931, Dako), or Rabbit IgG polyclonal isotype control (ab37415, Abcam) was applied at a similar concentration as the primary antibody.

2.18 | Statistical analysis

Data analysis was performed using Graph Pad Prism v8.1.1. All data was plotted as individual data points and median lines with biological repeats shown in individual colors. Normality was assessed by D'Agostino and Pearson and Shapiro–Wilk tests. Where data was shown to be normally distributed, data was compared using one-way ANOVA with Tukey's post hoc tests for pairwise comparisons between extraction protocols (rat, rabbit, dog), expansion media (rabbit, dog), and passage numbers (rabbit). Where extraction and expansion protocols had two variables investigated, two-way ANOVA with Tukey's post-hoc tests were utilized (pig, cow, human). Where repeated measures were made across passage number and surface coatings in human analysis, repeated measures mixed effects analysis with the Geisser–Greenhouse correction was performed. For percentage viability data which does not follow a normal distribution, Friedman test for paired data with Dunns Multi-comparison post hoc testing was performed. Human extraction studies involved two variables, and some data failed to satisfy normality testing; thus, a Wilcoxon matched pairs signed rank test was performed to determine significant differences between extraction durations.

2.19 | NP cells freezing and thawing methodology

Data were collected from each group who cryopreserve NP cells in liquid nitrogen. Data were categorized based on the freezing media components, freezing methods, and thawing methods and recommendations made across species from submitted protocols. Experimental testing of methodology was not felt necessary due to similarities in methods used for cryopreservation and expert review consensus.

3 | RESULTS

3.1 | Optimization of NP cell isolation from NP tissue

3.1.1 | Survey

Data were collected and collated from 25 groups worldwide who routinely extract and isolate NP cells from human IVD tissue and 18 groups who extract and isolate NP cells from different species (mouse, rat, rabbit, pig, dog, and cow). This analysis demonstrated that differences exist in the type, concentration, and timing of pronase and collagenase treatment used during cell isolation, as well as the size of the cell strainer (Figure 3). The majority of groups failed to report enzyme concentrations in units/ml and were reported in mg/ml. Of the 25 groups who submitted protocols for human NP cell isolation, 64% of groups reported using pronase with the most common concentration being 1.5–2 mg/ml for 1 h (Figure 3A,B). Four types of collagenases were reported for human NP cell extraction with type II being most common (Figure 3C) with a wide range of concentrations reported (from <0.5 to >2 mg/ml; Figure 3D). The most reported time for collagenase digest was >8 h or overnight, followed by 2–4 h (Figure 3E). Following digestion, 64% of groups passed their freshly digested human NP tissue through a cell strainer (Figure 3F), with 70 µm reported most commonly (Figure 3G).

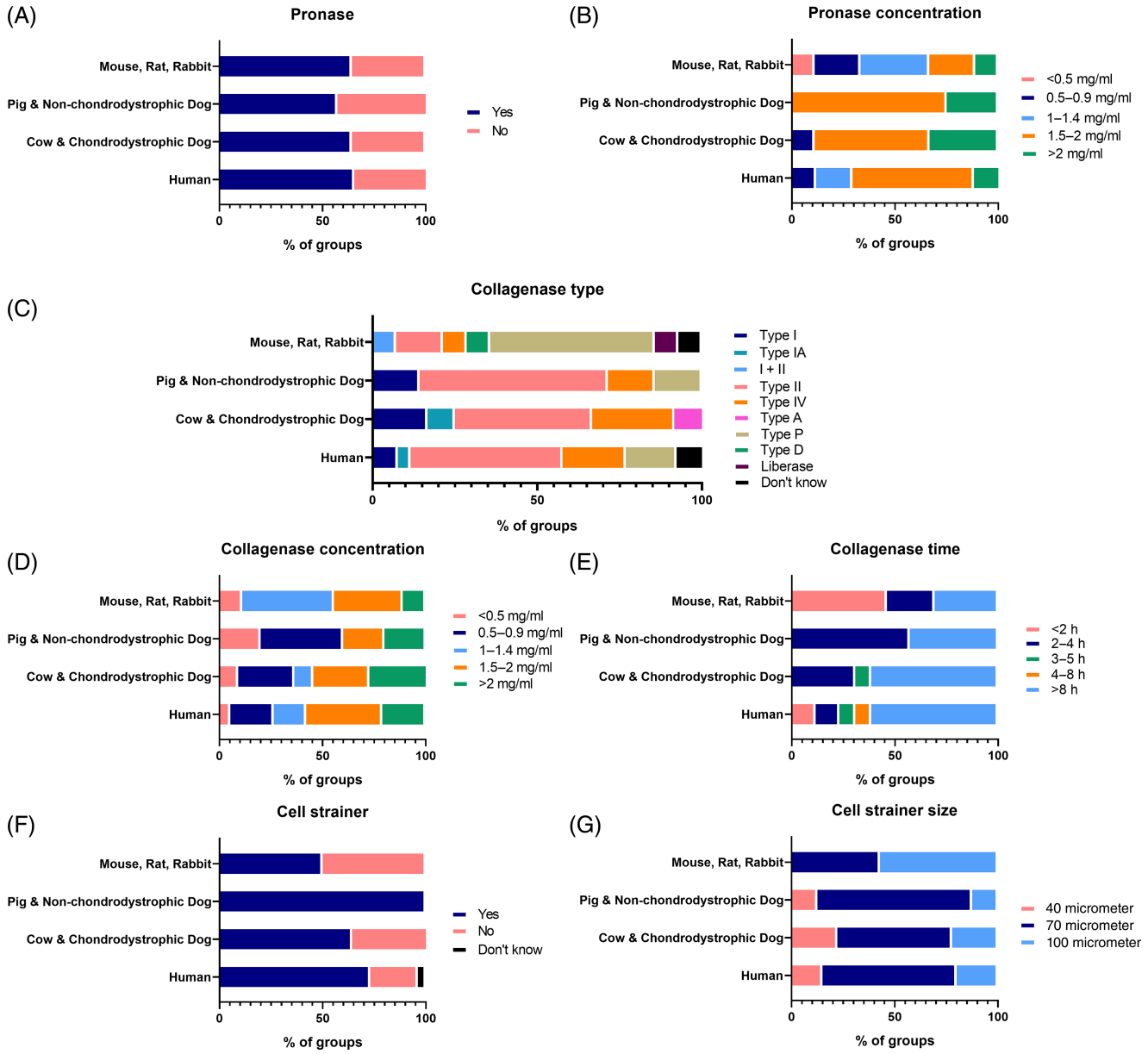


FIGURE 3 Survey data for extraction methods: NP cell isolation data was collected and analyzed from 36 groups worldwide on their NP cells extraction protocols. For analysis, animals were grouped as small animals (mouse, rat and rabbit) and large animals which retain the large vacuolated NC cells (pig and non-chondrodystrophic dogs) and those which lose their large vacuolated NCs (Cow and chondrodystrophic dogs). The isolation protocols were categorized based on the type, concentration, and timing of pronase and collagenase treatment, as well as the use and size of the cell strainer.

Of the 18 groups who submitted protocols for NP cell isolation from different animal species, 57% of groups reported using pronase with the most common concentration being 1.5–2 mg/ml for 1 h (Figure 3A,B). More types of collagenase, with six types reported across the species with type P most common in small animals (mouse, rat, and rabbit) but collagenase type II more common in larger animals (pig, cow, dog) (Figure 3C), with a wide range of concentrations utilized (Figure 3D). The most reported time for collagenase digest was >8 h or overnight although <2 h was reported commonly for small animals (Figure 3E). Following digestion, 65% of groups passed their freshly digested tissue through a cell strainer (Figure 3F), with 70 μm being the most common (Figure 3G).

3.1.2 | Harmonized methodology for cell isolation from NP tissue

To develop recommendations for cell isolation methodology key differential factors were tested experimentally to identify extraction methodology which obtained highest cell yield while maintaining key

phenotypic factors seen in vivo. Extraction methodologies were investigated from NP tissue of rats, rabbits, pigs, dogs, cows, and humans, with recommendations made to promote standardization.

3.2 | Small animal NP cell isolation

3.2.1 | Rat

Digestion methodology investigated the use of sequential or combined pronase and collagenase digestion together with investigation of two collagenase concentrations 100 U/ml (LO) or 400 U/ml (Hi). Approximately 2.8 ± 0.6 mg of tissue was isolated per disc (Figure 2C). Overall, combined digestion resulted in higher cell yields compared with the sequential digestion protocols with ~3–4 × 10^4 NP cells extracted per disc (Figure 2D). No significant differences were observed in cell viability for any of the protocols investigated (Figure 2E). Only one group had sufficient cell yield to perform gene expression analyses, and no differences in mRNA between digestion conditions were suggested by the data (Figure S1A). Histological evaluation, to determine if rat strain accounted for differences in cell count between groups, did not demonstrate apparent differences in cell density of the NP region between Wistar and Sprague–Dawley rats (Figure S1B).

3.2.2 | Rabbit

Digestion methodology investigated the use of sequential or combined pronase and collagenase type P digestion, together with differential pronase and collagenase treatments and digestion durations based on submitted protocols. Digestion Method 1: 30.84 U/ml pronase (45 min) and 0.16 U/ml collagenase P (1 h), sequentially. Digestion Method 2: 123.35 U/ml pronase (1 h) and 0.16 U/ml collagenase P (overnight), sequentially. Digestion Method 3: 61.68 U/ml pronase combined with 0.4 U/ml collagenase P for 2 h. Digestion Method 4: 246.71 U/ml pronase combined with 0.2 U/ml collagenase P for 1.5 h. Average cell counts yields of trypan blue excluded rabbit NP cells per disc (total number of cells/number of discs) were: digestion method 1: 1.21 ± 0.17 × 10^5 NP cells per disc; digestion method 2: 1.48 ± 0.24 × 10^5 NP cells per disc; digestion method 3: 1.56 ± 0.21 × 10^5 NP cells per disc; and digestion method 4: 1.08 ± 0.41 × 10^5 NP cells per disc (Figure 4). All procedures yielded similar numbers of isolated NP cells per disc with no significant differences observed ($P = 0.31$). Performing the pronase and collagenase digests at the same time (digestion methods 3 and 4) reduced the extra step of centrifugation to replace pronase with collagenase digest solutions (digestion methods 1 and 2). Digesting the NP tissue with the pronase and collagenase for 2 h (digestion method 3) instead of 1.5 h (digestion method 4) allowed for more removal of the gel-like tissue. While no significant difference was observed between final cell yield per isolated disc, due to reduced centrifugation steps and improved removal of tissue observed during the procedure digestion method 3 was selected as the recommended methodology.

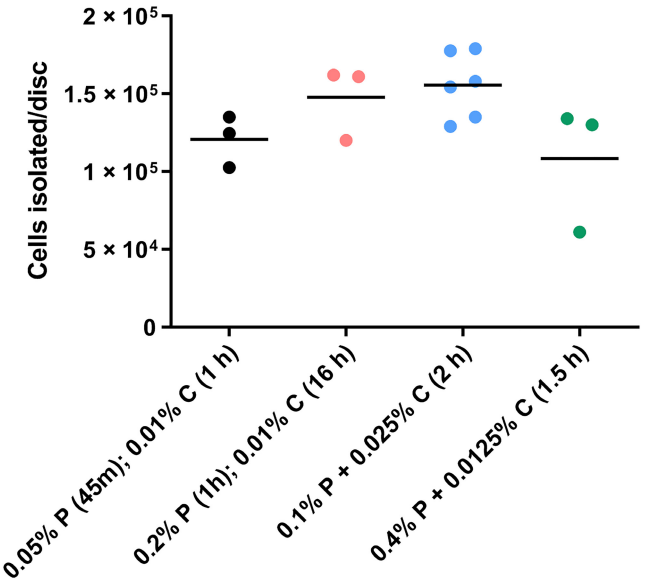


FIGURE 4 Cells were isolated from rabbit NP tissue using different methods: Number of NP cells isolated per disc from rabbit NP tissues following enzyme digestion conditions submitted by investigators, consisting of different concentrations of collagenase (C) and pronase (P) and different digestion times ($n = 3$–6). One-way ANOVA with Tukey's post hoc tests for pairwise comparisons between extraction protocols was performed to assess significance. All $P > 0.05$.

3.3 | Large animal NP cell isolation

3.3.1 | Pig

Comparing collagenase concentrations at the 4 h digestion period, there was evidence that the addition of pronase at 14 U/ml contributes to higher cell yields at either collagenase concentration (0.3 × 10^6 cells/gram of tissue versus 2.1 × 10^6 following 4 h of digestion), (Figure 5A). Comparing digestion periods of 4 h versus 16 h with different digestion methods, pronase contributed to higher cell yields following a 4 h digestion, however, a decrease was seen following 16 h digestion (Figure 5A). These differences in digestion conditions were associated with some differences in mRNA levels for all genes studied except *COL1A1* (Figure 5D). In brief, the addition of pronase to the digestion media at 4 h was associated with significantly lower levels of *ACAN* mRNA ($P < 0.05$; Figure 5B), *KRT18* ($P < 0.01$; Figure 5F), *T* ($P < 0.001$; Figure 5E), while a small decrease was also seen for *SHH* (Figure 5G), and *COL2A1* (Figure 5C) mRNA this did not reach significance, while *COL1A1* (Figure 5D) was not affected. The longer time period of digestion for conditions containing pronase was associated with higher levels for *COL2A1* (16 h vs. 4 h, C + P, $P < 0.001$), *T* ($P < 0.001$), and *SHH* ($P < 0.05$) but not for the other genes (Figure 5). In summary, since the highest cell yield was obtained with 4 h of digestion with 64 U/ml collagenase type II and 14 U/ml pronase, this was selected for cell isolation in all further studies although gene expression levels were reduced slightly under these conditions.

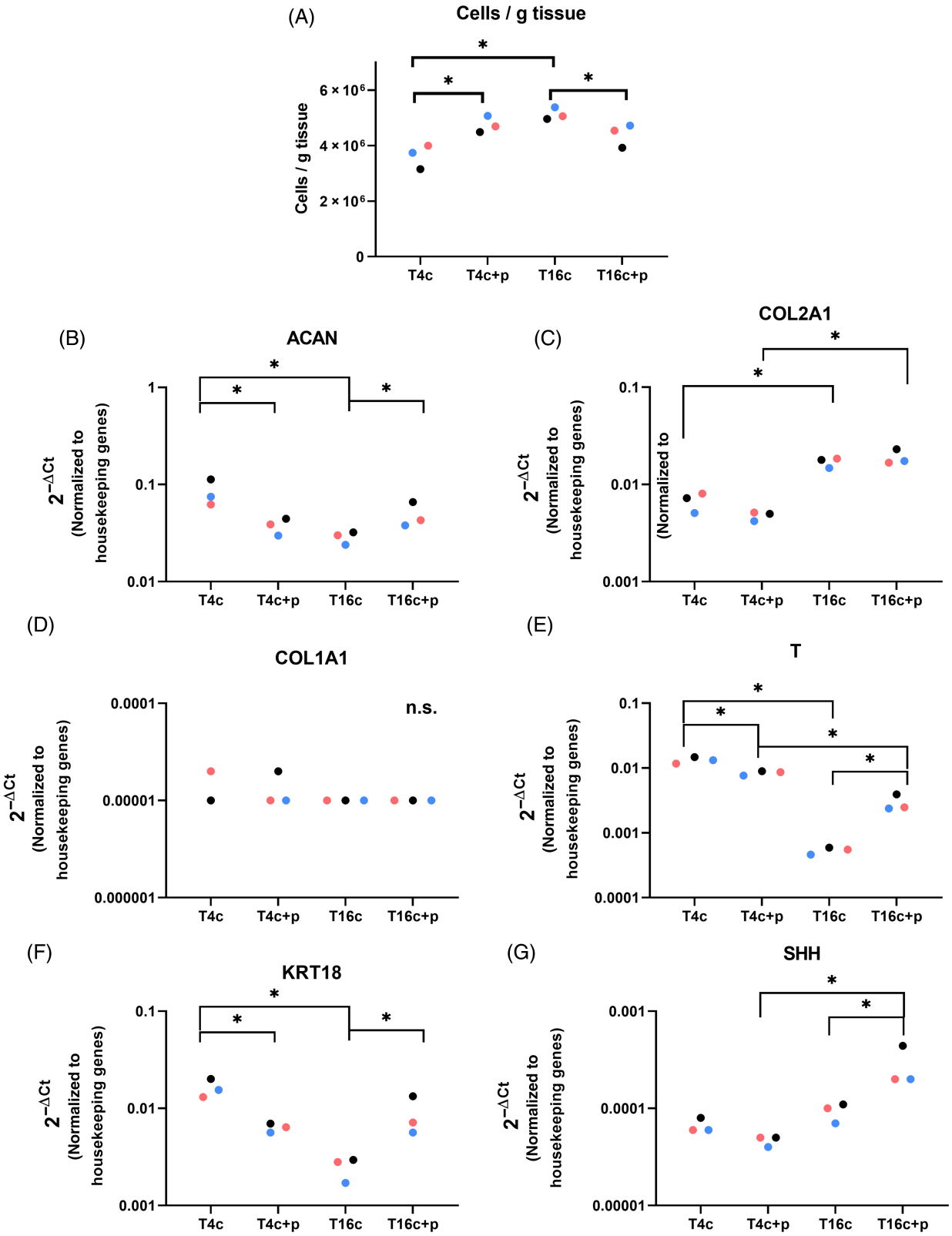


FIGURE 5 Cells were isolated from pig NP tissue using different methods. (A) Cell yields were measured for tissues digested with collagenase alone (C) compared with collagenase with added pronase (C + P) after a 4 h digestion period. (B) Cell yields were similarly measured under both isolation conditions at 16 h compared with 4 h. *$P < 0.05$, significant differences in cell yield were observed between conditions, ANOVA with Tukey's post hoc test. (C) Different conditions for cell isolation were associated with some differences in mRNA levels for cells at P0. Gene expression was quantified by RT-PCR by reporting $2^{-\Delta Ct}$ for each target where DCt was calculated from the difference in Ct for the target gene and the average of two housekeeping genes, 18S and B2M. Gene expression analysis analyzed with ANOVA with Tukey's post-hoc test *$P < 0.05$. Results pointed to generally high levels of gene expression for most targets (except brachyury), and the highest cell yield for tissues digested with C + P for the shorter time period.

3.3.2 | Dog

Dog NP tissue from chondrodystrophic dogs was digested for 4 or 16 h with collagenase type II, preceded or not with pronase digestion (30 min). Interestingly, cell viability appeared to increase when the tissue was pre-digested with pronase compared with NP tissue not pre-digested with pronase, although this failed to reach significance ($P = 0.347$; Figure 6A). Furthermore, live cell numbers appeared the highest after 16 h of collagenase digestion combined with pronase pre-digestion, although variability was high between donors and failed to reach significance ($P = 0.1417$) (Figure 6B). *ACAN* expression was increased and *COL2A1* expression decreased when NP tissue was digested with collagenase for 4 h instead of 16 h, with or without pronase pre-digestion ($P < 0.05$; Figure 6C,E). Moreover, the *BAX*/*BCL2* ratio (measure for apoptosis rate) was lower after 4 h versus 16 h of collagenase digestion ($P < 0.05$; Figure 6G,F). *COL1A1*, *KRT8*, and *KRT19* were observed but did not change between conditions (Figure 6D,H,I). Altogether, this indicates that 4 h instead of 16 h of collagenase digestion results in slightly improved NP cell phenotypic gene expression of the obtained dog NPCs.

3.3.3 | Cow

Cow NP cell extraction was investigated using collagenase types I and II, at low (64 U/ml) or high (256 U/ml) concentrations for 4 or 16 h

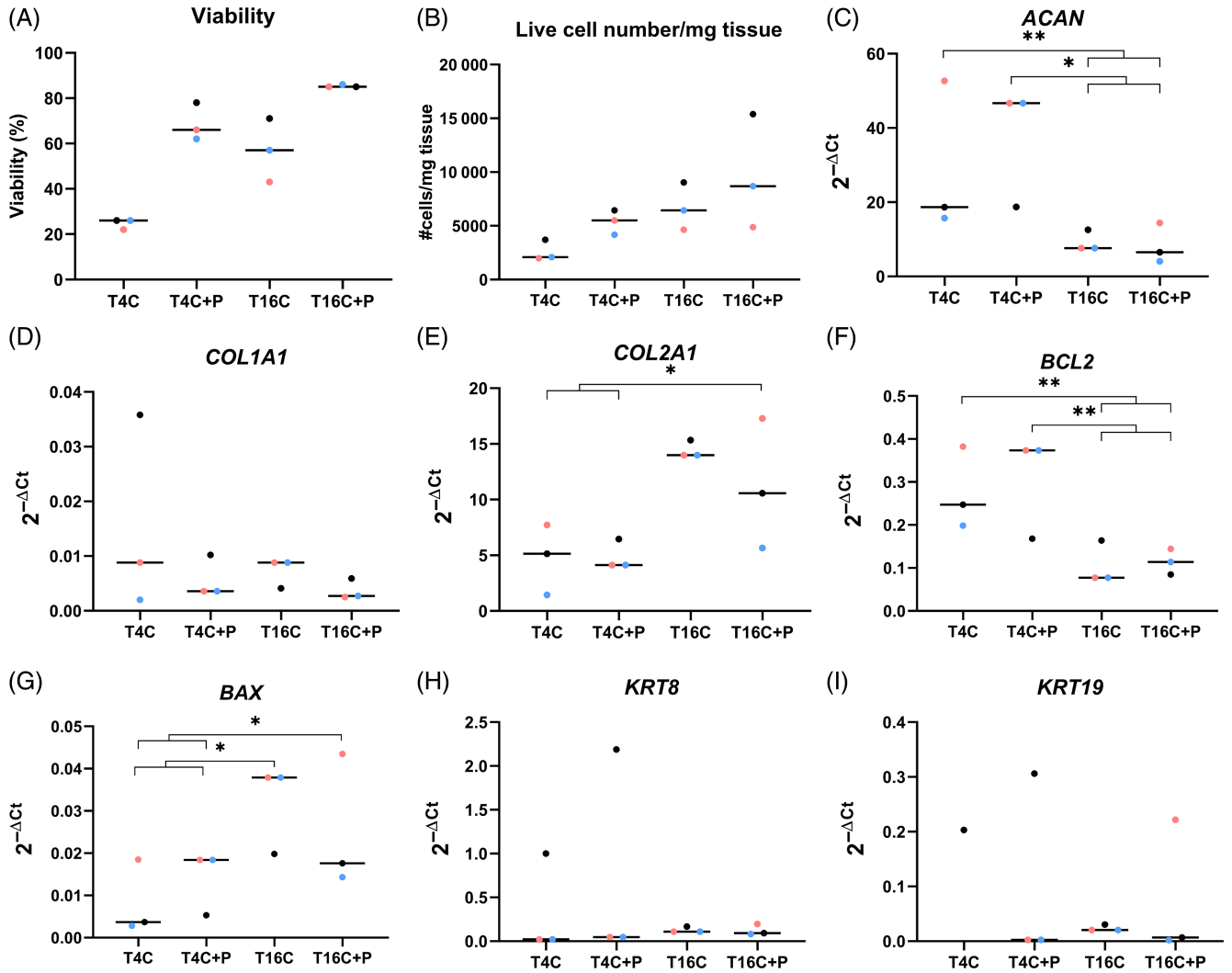


FIGURE 6 Cells were isolated from dog NP tissue using different methods. Tissues digested with collagenase alone (C) compared with collagenase with added pronase (C + P) after a 4 or 16 h digestion period. Viability (A) and live number of NP cells/g tissue (B) isolated from CD dog NP tissue following different collagenase type II digestion times (4 or 16 h) with/without 30 min of pronase predigestion. Gene expression for ACAN (C), COL2A1 (D), BAX (E), BCL2 (F), COL1A1 (G), KRT8 (H), and KRT19 (I) normalized for the mean expression of reference genes GAPDH, HPRT, and RPL19 immediately following extraction of NP cells from dog tissue. Digestion methods: T4c = 62.5 U/ml collagenase type II for 4 h; T4c + p = 5.25 U/ml pronase for 30 min followed by 62.5 U/ml collagenase type II for 4 h; T16c = 62.5 U/ml collagenase type II for 16 h; T16c + p = 5.25 U/ml pronase (30 min) followed by 62.5 U/ml collagenase type II for 16 h. Viability analysis was tested using Friedman test for paired data with Dunns multi-comparison post hoc testing. Live cell number and gene expression analysis was analyzed with ANOVA with Tukey's post hoc test *$P < 0.05$. $n = 3$ dogs (A and B), in triplicates (D–I).

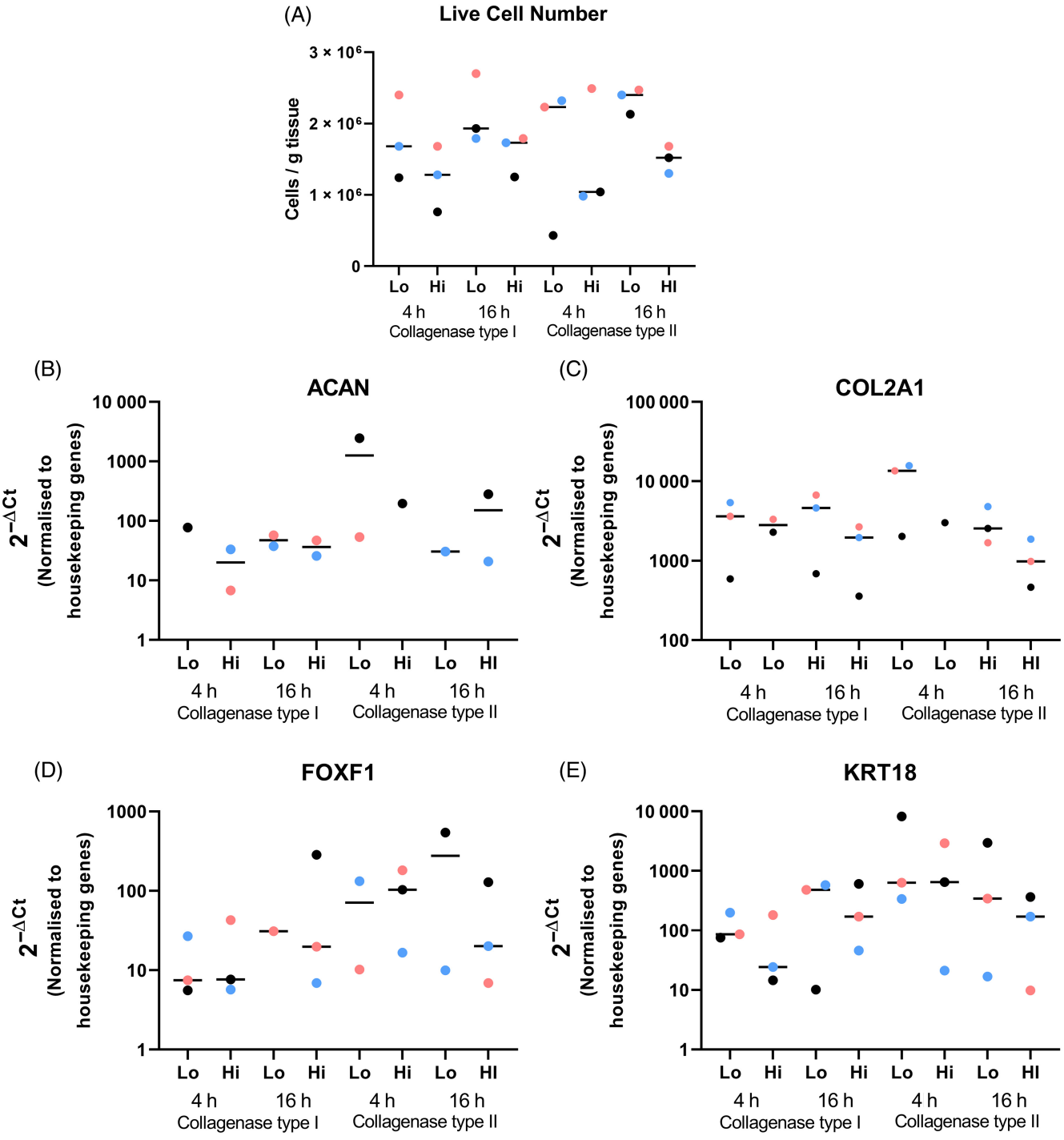


FIGURE 7 Cells were isolated from cow NP tissue using different methods. Tissues digested with collagenase type I or type II at 64 U/ml (Lo) or 256 U/ml (Hi) after a 4 or 16 h digestion period. (A) Number of NP cells isolated from cow NP tissue following different collagenase type, concentration, and digestion time. (B–E) Gene expression for COL2, ACAN, KRT18, and FOXF1 in cells immediately following extraction of NP cells from cow tissue with different collagenase type, concentration and digestion time. Two-way ANOVA with Tukey's post-hoc tests utilized to identify statistical effects. All $P > 0.05$.

digestion period. There were no significant differences in the number of cells retrieved or viability between collagenase type, concentration, and digestion time from cow NP tissues ($P > 0.05$) (Figure 7A). Following isolation, gene expression of *COL1A1*, *PAX1*, *IL-1*, *MMP3*, *MMP13*, and *ADAMTS-4* was not detected (data not shown), while *ACAN*, *COL2A1*, *FOXF1*, and *KRT18* were expressed, there were no significant differences between groups ($P > 0.05$) (Figure 7B–E). *COL2A1* was decreased in cells digested in collagenase type II at 256 U/ml for 16 h compared with 4 h but this failed to reach significance (Figure 7B).

3.3.4 | Human NP cell isolation

No significant differences in the number of cells or viability retrieved from human NP tissue following 64 U/ml collagenase type II digestion for either 4 or 16 h, suggesting that all cells are released from the tissue following 4 h digestion ($n = 5$; Figure 8A). Furthermore, no significant differences were seen in the gene expression of NP markers between 4 or 16 h digestion in collagenase type II immediately following extraction although a trend of reduced *ACAN*, *COL2A1*, and *COL1A1* expression was observed with longer digestion period ($P > 0.05$) (Figure 8B–F).

3.4 | NP cell culture media

Following a preliminary review of the 36 groups whose protocols were submitted for expansion media and 17 protocols for 3D re-differentiation media, the addition of several supplements to the basal media were identified (Figure 9). Both DMEM and DMEM/F12 were the most commonly used basal media across all species, although Ham/F12 and αMEM were reported by some groups in smaller animals (Figure 9A). Most protocols reporting using pyruvate within the media, with the exception of cow and chondrodystropic dogs, with 110 mg/ml being the most common concentration (Figure 9B,C). The majority of groups included antibiotics and fungizone, and the majority of groups reporting for cow, chondrodystrophic dogs, and human NP cells used ascorbic acid, with an increase in number of groups using ascorbic acid seen in 3D culture (Figure 9D). However, very few groups culturing mouse, rat, rabbit, pig, or non-chondrodystrophic dog NP cells used ascorbic acid although some of these groups utilized αMEM which already includes L-AA (Figure 9D). For those groups who reported using ascorbic acid a range of concentrations were reported, with ~0.3 mM (50 μg/ml) the most common (Figure 9E). The type of ascorbic acid also varied between the less stable L-AA which groups mainly added fresh to account for the low stability, while some groups utilized L-AA 2 phosphate which is more stable in culture media (Figure 9F,G). The addition of growth factors was reported by very few groups and included either FGF-2, platelet-derived growth factor (PDGF), and transforming growth factor (TGF) with FGF-2 being the most commonly used (Figure 9H). While most groups reported using 10% FCS and high glucose media with 4.5 g/L being the most common across all species within expansion media, when transferring to 3D culture most groups switched to low glucose media (Figure 9I,J) and 25% of groups reported FCS alternatives (Figure 9I). The oxygen levels varied from 3% to 21% with normoxic

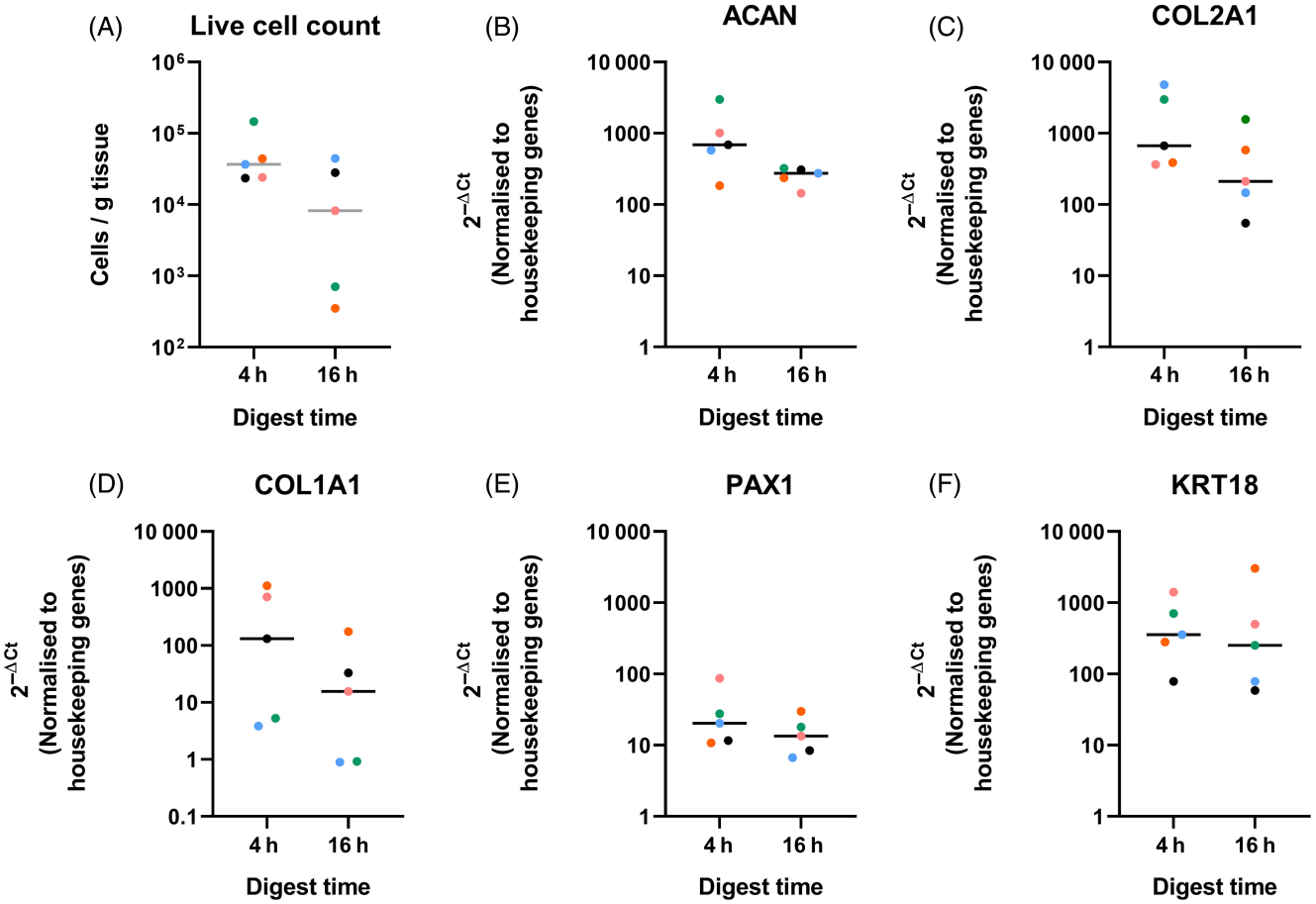
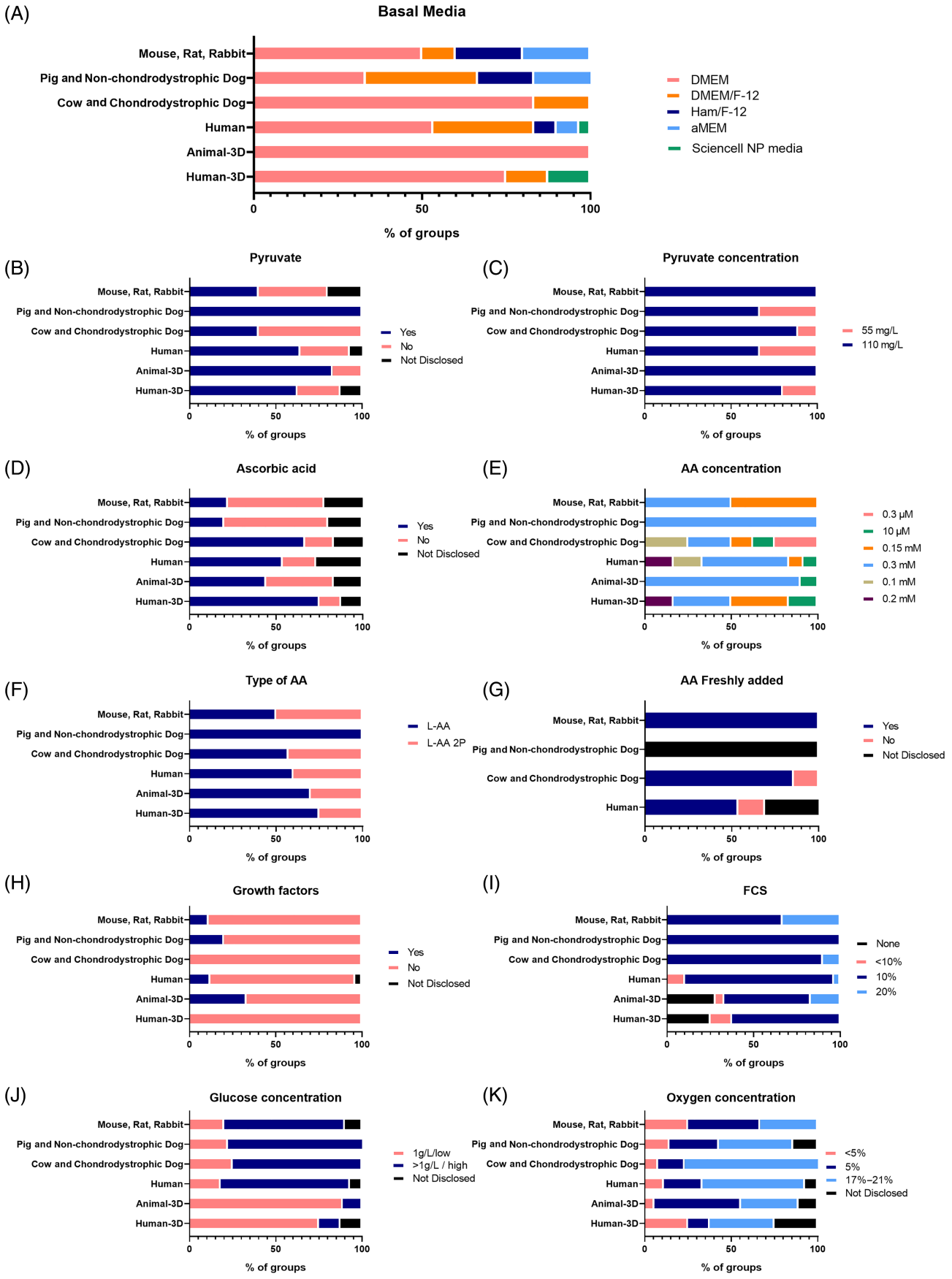


FIGURE 8 Cells were isolated from human NP tissue using different methods. Human NP tissue was digested with 64 U/ml Collagenase type II for either 4 or 16 h. (A) Number of live cells per gram of tissue. (B–F) Gene expression for ACAN, COL2A, PAX1, KRT18, and COL1A1 in cells immediately following extraction of NP cells from Human NP tissue for 4 or 16 h 64 U/ml collagenase type II. [a]Wilcoxon matched pairs signed rank test utilized to investigate influence of extraction duration. *$P < 0.05$.



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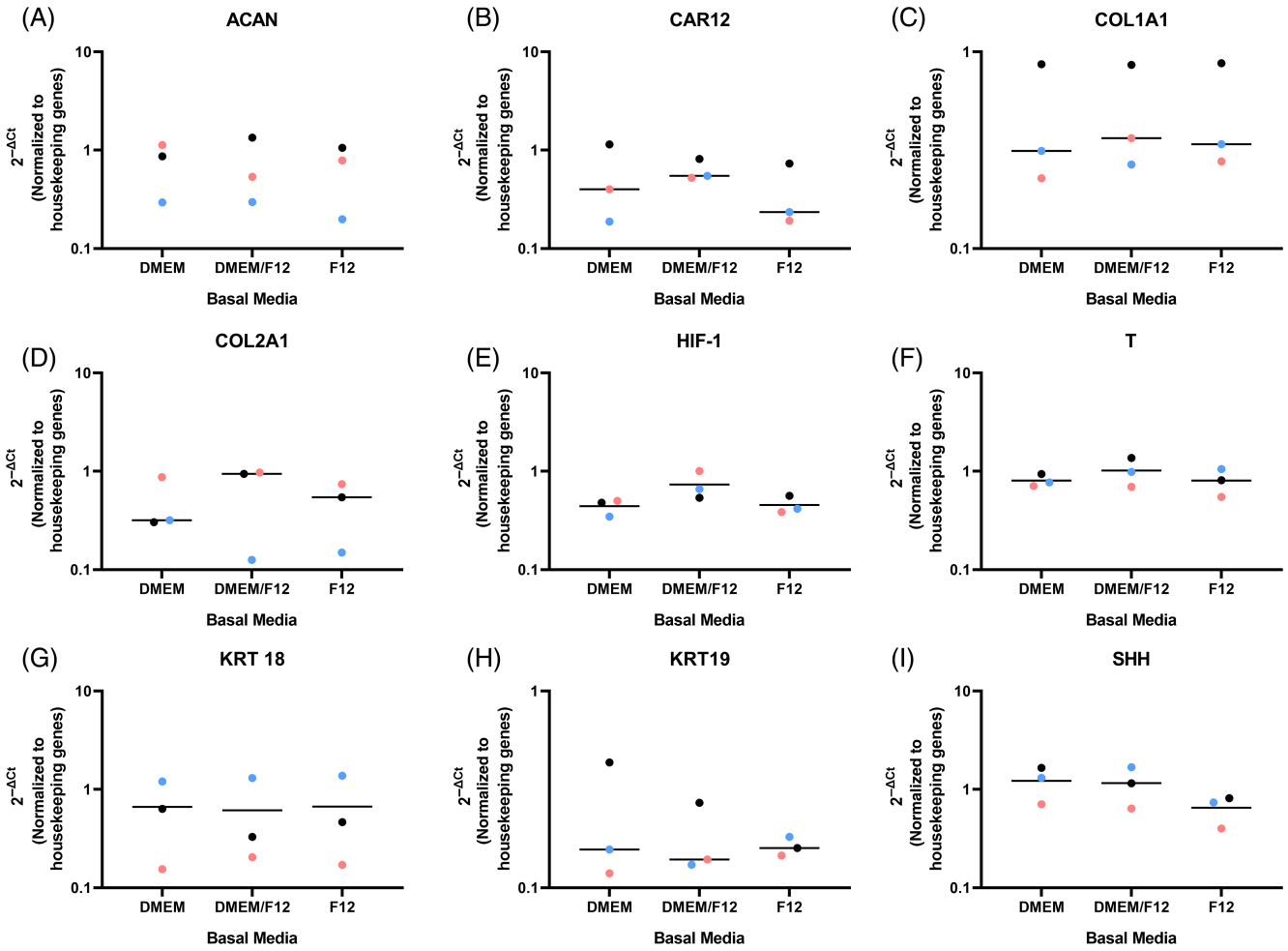

FIGURE 10 Phenotypic analysis of rabbit NP cells cultured in different basal media. Freshly isolated rabbit NP cells were plated and cultured in complete DMEM/F12 for a week and in complete DMEM/F12, F12, or DMEM media for another week before collection. Gene expression levels of ACAN, CAR12, COL1A2, COL2A1, HIF1A, KRT18, KRT19, SHH, and T were analyzed.

(17%–21% O_2) culture being the most commonly reported condition for the expansion of large animals and human NP cells, lower oxygen conditions more representative of physioxia (5% O_2) were reported for small animals and in animal and human NP cell cultures once in 3D (Figure 9K).

3.5 | Basal media optimization

3.5.1 | Rabbit

No significant differences in expression levels of phenotypic NP genes were observed when comparing rabbit NP cells cultured in the different media (hgDMEM/F12, hgF12, or hgDMEM) for 1 week (Figure 10).

3.5.2 | Cow

Expansion of cow NP cells ($n = 3$) in complete hgDMEM or hgDMEM/F12 showed no difference in cumulative population doubling rates from P0 to P3 between basal media (Figure 11A). No significant differences in mRNA expression levels of *ACAN*, *COL2A1*, *COL1A1*, *KRT18*, or *FOXF1* were seen either in monolayer or alginate culture between DMEM and DMEM/F12 ($P > 0.05$) (Figure 11B,E,F). *PAX1* was not detectable under any culture conditions (data not shown). mRNA expression for *ACAN* and *KRT18* was induced and *COL1A1* decreased in alginate compared with monolayer culture, although statistical analysis not possible due to lack of expression in some samples (Figure 11B,D,E). *COL2A2* was decreased below detectable limits

FIGURE 9 Survey data for culture media utilized for expansion and 3D culture of NP cells. NP cell expansion data was collected and analyzed from 36 groups worldwide on their NP cells culture protocols. For analysis, animals were grouped as small animals (mouse, rat, and rabbit) and large animals which retain the large vacuolated NC cells (pig and non-chondrodystrophic dogs) and those which lose their large vacuolated NCs (cow and chondrodystrophic dogs). The 3D culture media protocols received from 17 groups worldwide, animal protocols represented separately ($n = 9$) from human protocols ($n = 8$). Cell culture media was categorized based on the type of media, FCS concentration (or lack of), inclusion of pyruvate, ascorbic acid (and type), use of growth factors O_2 concentration, and glucose concentration.

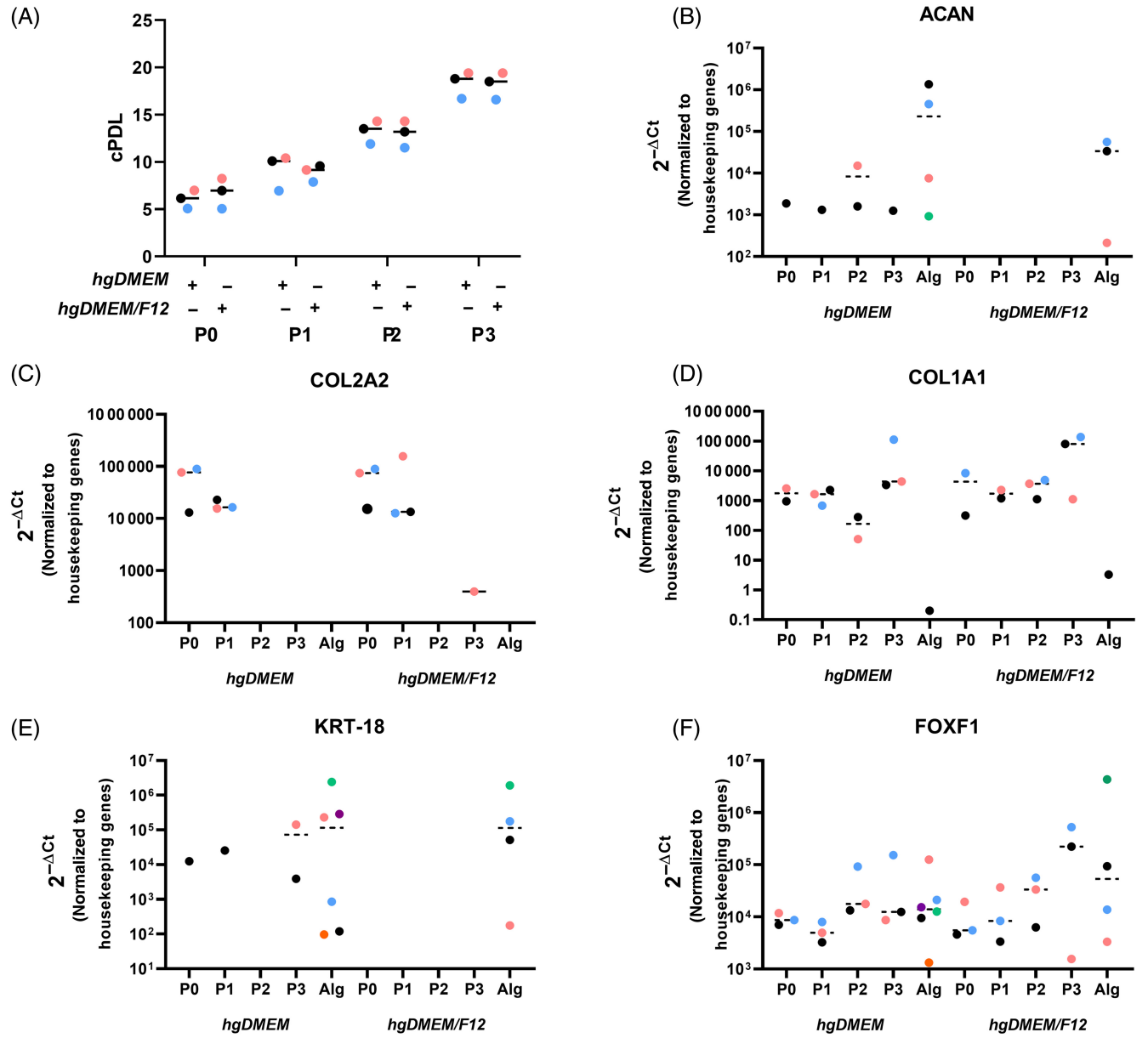

FIGURE 11 Phenotypic analysis of cow NP cells cultured in different basal media. (A) Directly extracted cow NP cells were cultured in either complete hgDMEM or hgDMEM/F12 media. To compare NP cells growth rate, cumulative population doubling level (cPDL) was calculated for two different culture conditions. No significant difference was observed between NP cells growth in DMEM as compared with DMEM/F12 media. (B–F) To test for phenotypic changes, directly extracted cow NP cells were cultured in complete DMEM or DMEM/F12 up to passage 3 or in alginate culture. Cultured cells were harvested and relative gene expression of ACAN, COL2A1, COL1A1, KRT18, and FOXF1 were analyzed and compared between different culture conditions. Kruskal–Wallis and Dunns post hoc testing showed no significant difference $P > 0.05$.

during monolayer culture with increased passage which was not regained during alginate culture (Figure 11C). *FOXF1* was not altered during expansion in monolayer or culture in alginate (Figure 11F).

3.5.3 | Human

For human NP cell expansion, a direct comparison of hgDMEM and hgDMEM/F12 media with or without laminin-coated culture surfaces was performed. There were no significant differences in cell count (Figure 12A) after 1 week in culture with DMEM or DMEM/F12, but there were significant differences in *ACAN* and *COL2A1* expression due to basal media (Figure 12B,C). There were no significant differences in cell count between uncoated tissue culture plastic, 2 µg/ml laminin, and 20 µg/ml laminin (Figure 12A). However, there were significant differences in expression of *ACAN* and *COL2A1* due to different laminin coating concentrations when cells were cultured in hgDMEM (Figure 12B,C).

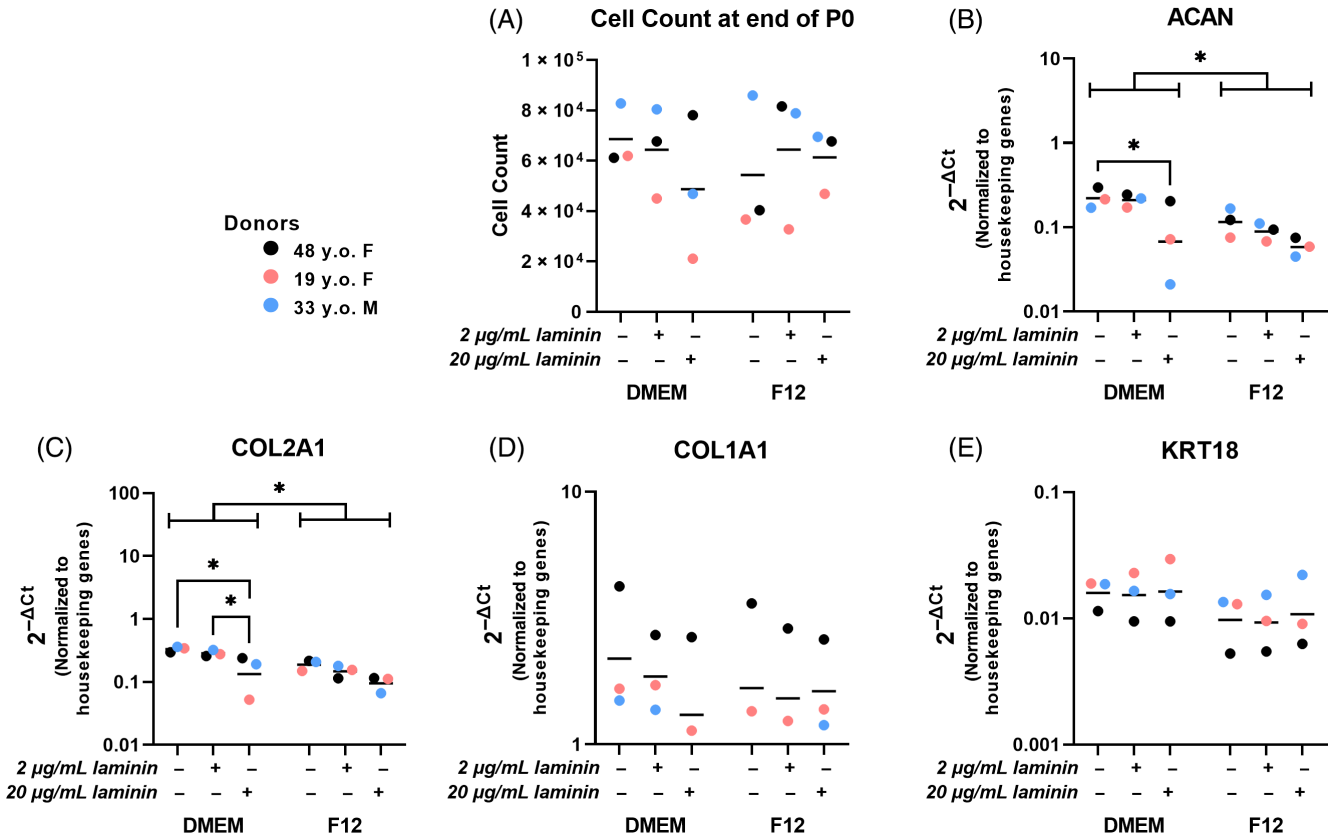

FIGURE 12 Phenotypic analysis of human NP cells cultured in different basal media ± Laminin coated tissue culture plastic. The effects of different basal media (DMEM or DMEM/F12) were assessed via human NP cells from recently deceased organ donors ($n = 4$) cultured in either F12 or high glucose DMEM with 10% FCS and on either uncoated tissue culture plastic, 2 µg/ml laminin, or 20 µg/ml laminin until the end of P0 for assessment of cell count (A) and qRT-PCR (B-E). All qRT-PCR was normalized to GAPDH and RNA45S5. Two-way ANOVA with Tukey's post hoc tests. *$P < 0.05$.

3.6 | Effect of passage number

3.6.1 | Rabbit

Compared with rabbit NP cells from P0, NP cells from P1 and P2 expressed significantly less *ACAN*, *COL2A1*, T, *KRT19*, and *SHH* in normoxic conditions ($P < 0.02$, Figure 13A,B,F,H,I). Gene expression of *KRT18* decreased but the decrease was not significant in normoxic conditions ($P = 0.068$) (Figure 13G). Gene expression of *COL1A1*, *CAR12*, and *HIF1α* increased at later passage numbers in normoxia, but these increases were not significant.

3.6.2 | Pig

Pig NP cells were expanded out to passage 3 with results showing the population doubling time dropped significantly from over 15 h for P0 to <10 h for P1 and P2 (Figure 14A). There was evidence of a substantial and significant drop in gene expression for *KRT18 and T* following the first cell passage that persisted for P1 and P2 ($P < 0.05$; Figure 14E,F). There was also a smaller yet significant decrease in gene expression for *ACAN* ($P < 0.05$), and while *COL2A1* also decreased this failed to reach significance (Figure 14B,D). While an increase in *COL1A1* gene expression was observed at P2 (Figure 14C) ($P < 0.05$). While differences in cell expansion media were not studied, differences in substrate coatings of Geltrex™ versus gelatin were compared. No significant differences were observed between substrate coatings in all marker genes, indicating that gelatin coating provides a suitable surrogate for Geltrex™ in pig cells.

3.6.3 | Human

Changes in NP cell phenotype in monolayer culture were investigated over time up to four passages. The initial acclimation period during which the NP cells adapted to monolayer culture prior to the first passage was about a week (7.75 days ± 2.49), while subsequent passages were significantly faster as determined by repeated measures one-way ANOVA (Figure 15A). The cumulative population doubling level (cPDL) approximately matched the passage number for the first two passages (Figure 15B, passage 0 mean cPDL = 0.96, passage 1 mean cPDL = 2.15) but was less well-matched later (passage 2 mean

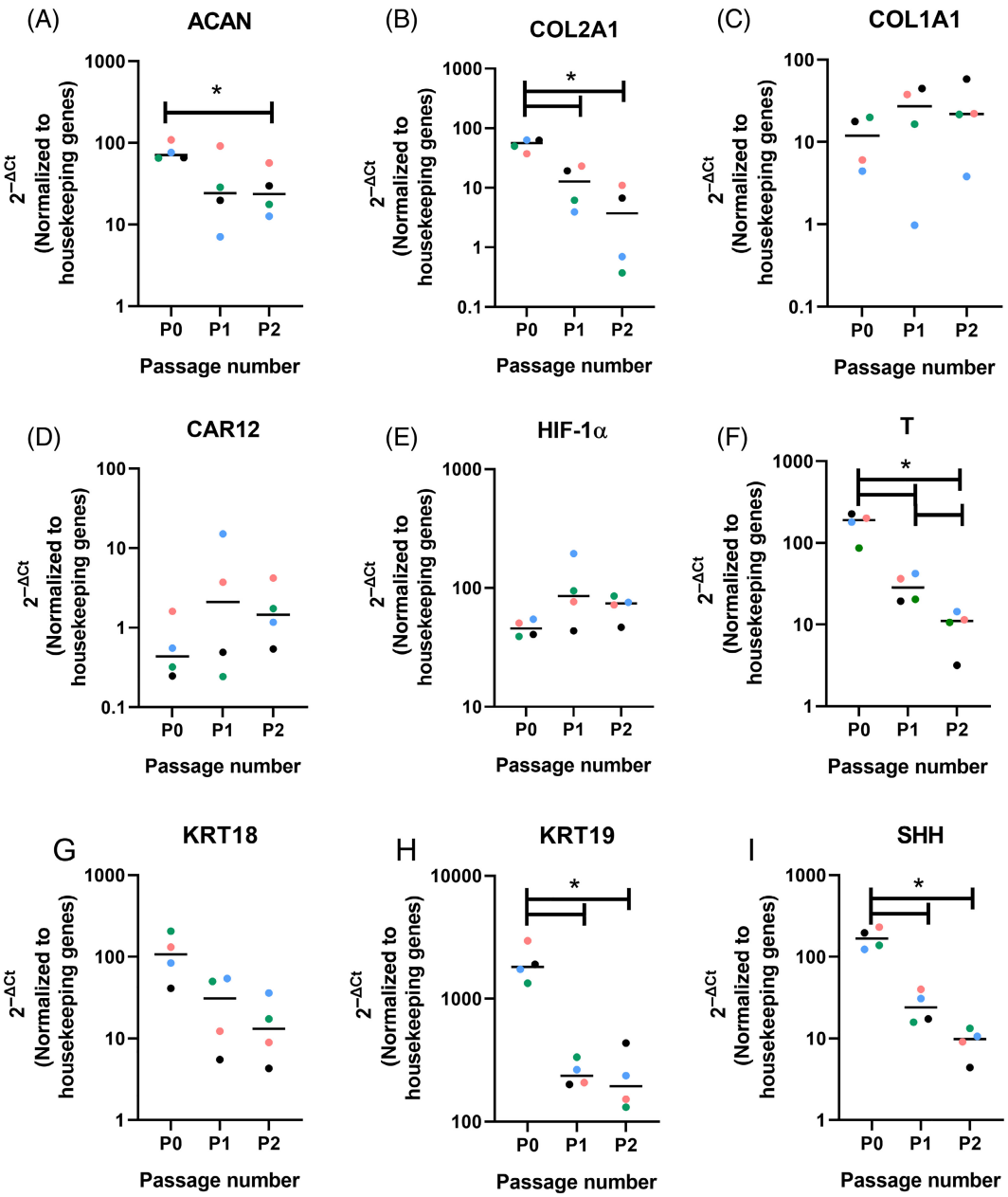

FIGURE 13 Effect of expansion on rabbit NP cell phenotype. Rabbit NP cells expanded to different passage numbers, RNA extracted at the end of P0, P1, P2 to assess gene expression levels of ACAN, COL1A2, COL2A1, CAR12, HIF1A, T, KRT18, KRT19, and SHH. One-way ANOVA with Tukey's post hoc tests to assess effects between passages. *$P < 0.05$.

cPDL = 2.38). The gene expression of the human NP cells was substantially affected by passage number/cPDL. Expression of *ACAN*, *COL2A1*, *and KRT18* were significantly decreased, while *COL1A1* was significantly increased with increased passage number based on repeated measures mixed effects analysis (Figure 15C–F). Significant differences between individual passages were observed for all genes of interest when the uncoated and Geltrex™-coated conditions were combined (Figure 15C–F).

Taken together, plating the NP cells from all species in monolayer culture was associated with a trend toward de-differentiation of the NP phenotype toward a more fibroblastic phenotype.

3.7 | Alternatives to FCS in expansion media

3.7.1 | Dog

To test whether dog NPCs were able to expand without FCS, they were expanded in hgDMEM + Glutamax, 1 ng/ml FGF-2, 0.1 mM Asap, 0.5% Fungizone, 1% ITS + premix, 0.04 mg/ml L-proline, and 1.25 mg/ml HSA with T175 flasks that were coated with 5 µg/ml human rLaminin-521 (Corning, 354221). After 14 days, however, no cells had attached and they all died in culture, indicating that dog NPCs cannot be expanded on laminin without FCS. As such decreased

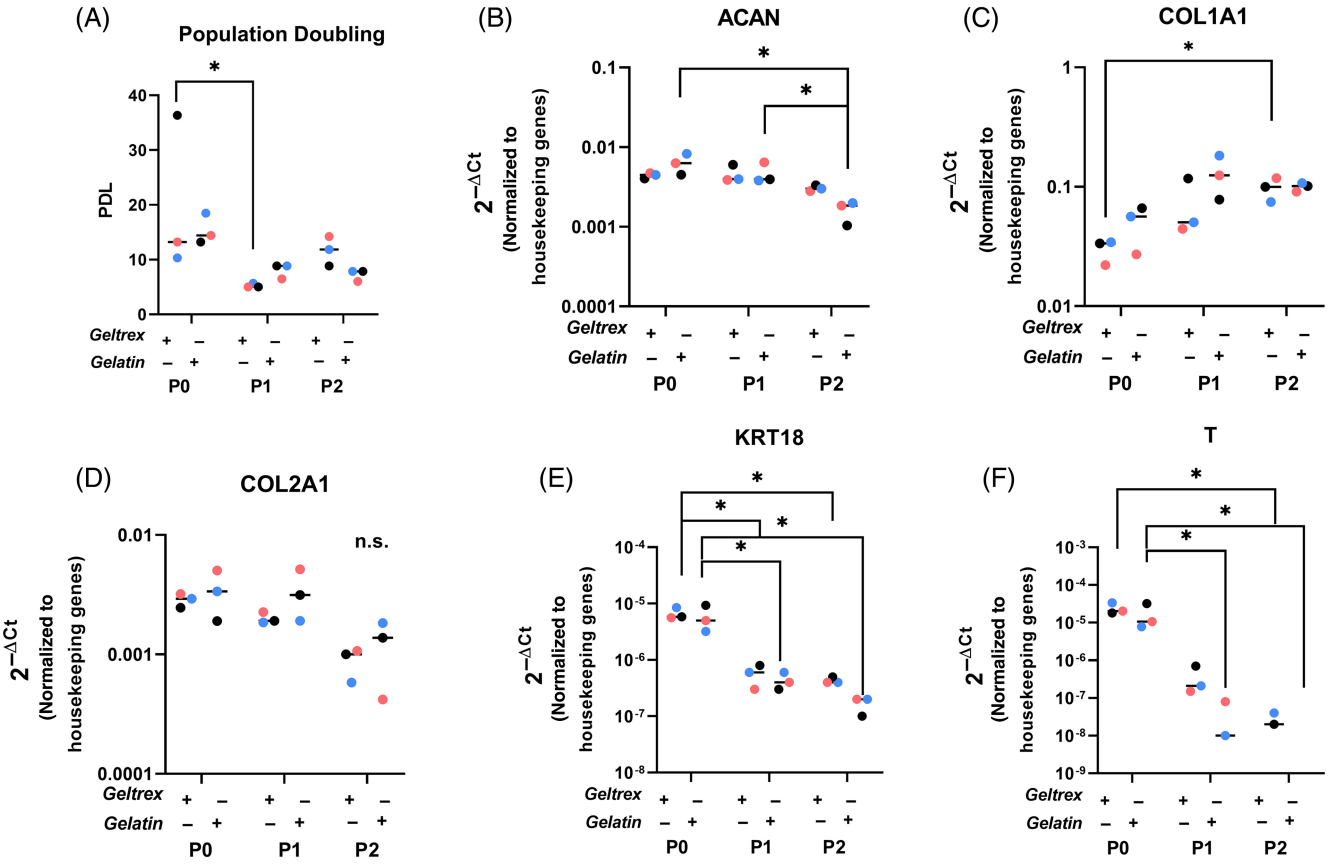

FIGURE 14 Effect of expansion on pig NP cell phenotype. Pig NP cells isolated following digestion with C + P (4 h) and plated upon flasks coated with gelatin or GeltrexTM. RT-PCR was performed on mRNA extracted from cells immediately upon isolation (P0) and following expansion to P3. (A) Cumulative population doubling times (cPDL, c) decreased significantly from P0 to P2 on both substrates (*$P < 0.005$, ANOVA). (B) RT-PCR was performed to calculate gene expression relative to the average of 18 s and B2M mRNA and reported as $2^{-\Delta Ct}$. In general, there was no evidence of differences in mRNA for any target between gelatin and Geltrex culture conditions. mRNA levels for most targets decreased after P0–P1 levels, except for COL1A1. ANOVA with Tukey's post-hoc test *$P < 0.05$.

FCS was tested within expansion media to determine whether dog NPCs could be expanded with a lower concentration of FCS than usual, and whether FGF-2 would improve the performance of the dog NPCs. Three different expansion culture media were tested on dog NPCs: 10% FCS, 5% FCS + FGF, and 5% FCS. The cPD of the 10% FCS condition (both after 4 and 16 h of tissue digestion with collagenase) appeared to be slightly higher compared with the other expansion conditions (Figure 16A), although donor variability was high. Gene expression analysis indicated no differences in *COL1A1*, *CCND1*, *KRT8*, and *KRT19* expression between conditions (Figure 16B,E–G). *ACAN* and *COL2A1* expression was, however, significantly higher in NPCs expanded in 10% FCS compared with the other conditions (Figure 16C,D). Altogether, these results indicate that dog NPCs can be expanded in 5% instead of 10% FCS, but that ECM-related gene expression is considerably decreased. Moreover, 1 ng/ml FGF-2 did not significantly affect dog NPC expansion.

After expansion in the three different conditions, dog NPCs were re-differentiated in alginate beads in culture medium with and without FCS, to determine whether dog NPCs could be re-differentiated without the usual supplementation of 10% FCS. In total, six different conditions were tested (three different expansion media and two different redifferentiation media): (1) $E_{10\%}$ R_{FCS}, (2) $E_{10\%}$ R_{noFCS}, (3) $E_{5\%+FGF}$ R_{FCS}, (4) $E_{5\%+FGF}$ R_{noFCS}, (5) $E_{5\%}$ R_{FCS}, and (6) $E_{5\%}$ R_{noFCS}.

Gene expression analysis indicated only minor differences between the six conditions: only *COL2A1* expression was significantly lower in $E_{5\%}$ R_{noFCS}-treated samples compared with $E_{10\%}$ R_{FCS}- and $E_{5\%}$ R_{FCS}-treated alginate beads (Figure S2). *KRT8* expression was low in all conditions and *KRT19* expression was not detected (Figure S2A). *COL2A1* expression was significantly increased after redifferentiation, whereas the expression of *COL1A1* was significantly decreased (Figure S2).

The DNA content of the cell fraction of the re-differentiated NPC alginate beads was significantly ($P = 0.04$) decreased at day 14 compared with the start of the redifferentiation (day 0) (Figure 17A). Unexpectedly, there was also DNA detected in the matrix fraction of the alginate beads (Figure 17B). Although not significant, this was especially seen in the NPCs re-differentiated without FCS (Figure 17B). When the DNA content of the cell and matrix fraction were combined these trends were still present (Figure 17C).

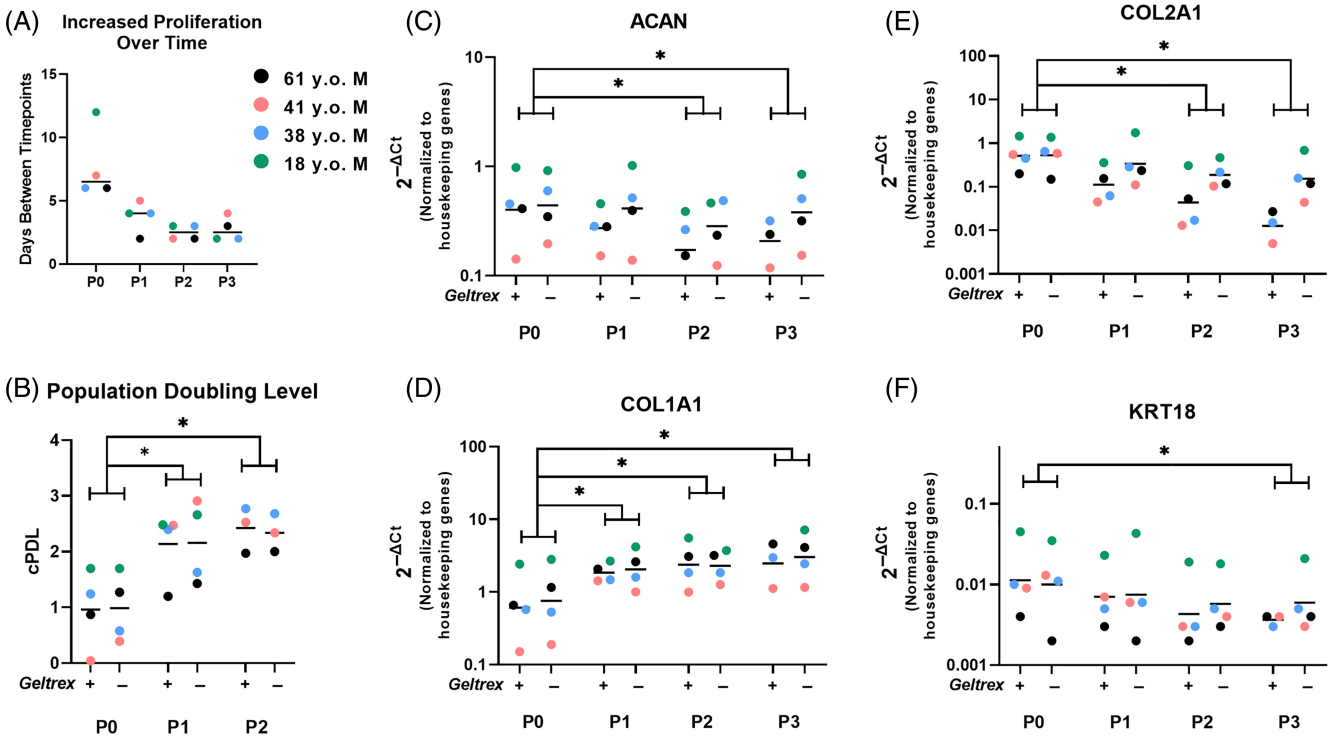

FIGURE 15 Effect of expansion on human NP cell phenotype. Human NP cells from recently deceased organ donors ($n = 4$) were cultured in high glucose DMEM with 10% FCS on either Geltrex-coated or uncoated tissue culture plastic for 4 passages to investigate changes over time in the number of days between passages (A), cumulative population doubling level (cPDL, B), and qRT-PCR (C–F). All qRT-PCR was normalized to GAPDH and RNA45S5. Repeated measures mixed effects analysis with the Geisser–Greenhouse correction and repeated measures one-way ANOVA with Tukey's post hoc tests *$P < 0.05$.

The GAG content of the cell fraction of the re-differentiated NPC alginate beads was significantly decreased at day 14 compared with the start of the redifferentiation independent of the expansion condition (day 0) (Figure 17D). Redifferentiation without FCS seemed to result in a lower GAG content in the cell fraction, although not significant (Figure 17D). No significant differences were detected in GAG content of the matrix fraction of the NPC alginate beads, also not after correction for the signal of the empty beads (Figure 17E). When the GAG content of the cell fraction and the corrected GAG content of the matrix fraction were combined, the GAG content of the NPCs expanded in 5% FCS was significantly decreased following alginate culture for 14 days compared with day 0 independent of the addition of FGF-2 during expansion (Figure 17F).

When the GAG content of the cell fraction was corrected for the DNA content of the cell fraction, the GAG content at day 14 was significantly higher in the re-differentiated NPCs with FCS compared with day 0 after expansion without FGF-2 (Figure 17G). The combined GAG content (cell fraction together with matrix fraction corrected for empty beads) corrected for the combined DNA content (cell fraction and matrix fraction together) was also significantly increased in the NPCs re-differentiated for 14 days with FCS compared with day 0 after expansion in 5% FCS without FGF-2 (Figure 17I). This increase was not detected for the GAG content of the matrix fraction corrected for the DNA content (Figure 17H).

Histology showed healthy, rounded, single cells at day 0 (Figure 18A), with more cell clusters after 14 days of differentiation in all six conditions. Aggrecan deposition was especially high at day 0 (inside the single cells) and at day 14 intercellular in the cell clusters, but not in the single cells (Figure 18B). Type I collagen deposition was not encountered at day 0 (Figure 18B). After 14 days, type I collagen appeared slightly more extensively deposited in the clusters of the conditions that were expanded in 10% FCS. Type II collagen deposition was also not detected at day 0, but at day 14 mainly in the redifferentiation conditions that contained FCS (Figure 18B). Almost no PAX1 positivity was detected at day 0 (Figure 18B), indicating no difference between the expansion conditions. After 14 days, there was more immunopositivity of PAX1 detected in the NPCs re-differentiated with FCS (Figure 18B). Keratin8 and Keratin19 (data not shown) immunopositivity was not detected in any condition.

3.8 | Human

To determine whether culture conditions reported by groups for 3D culture which omit the need for FCS could also be utilized for monolayer expansions, we went on to assess the effect of FGF-2 with FCS alternatives such as ITS-X and Albumax compared with standard FCS in human NP cultures. However, similarly to what was seen for dog NP cells, very low cell adherence was observed in the absence of FCS. Thus, subsequent studies tested fibronectin and Geltrex™ coatings to investigate improved cell adherence, and while small numbers of cells

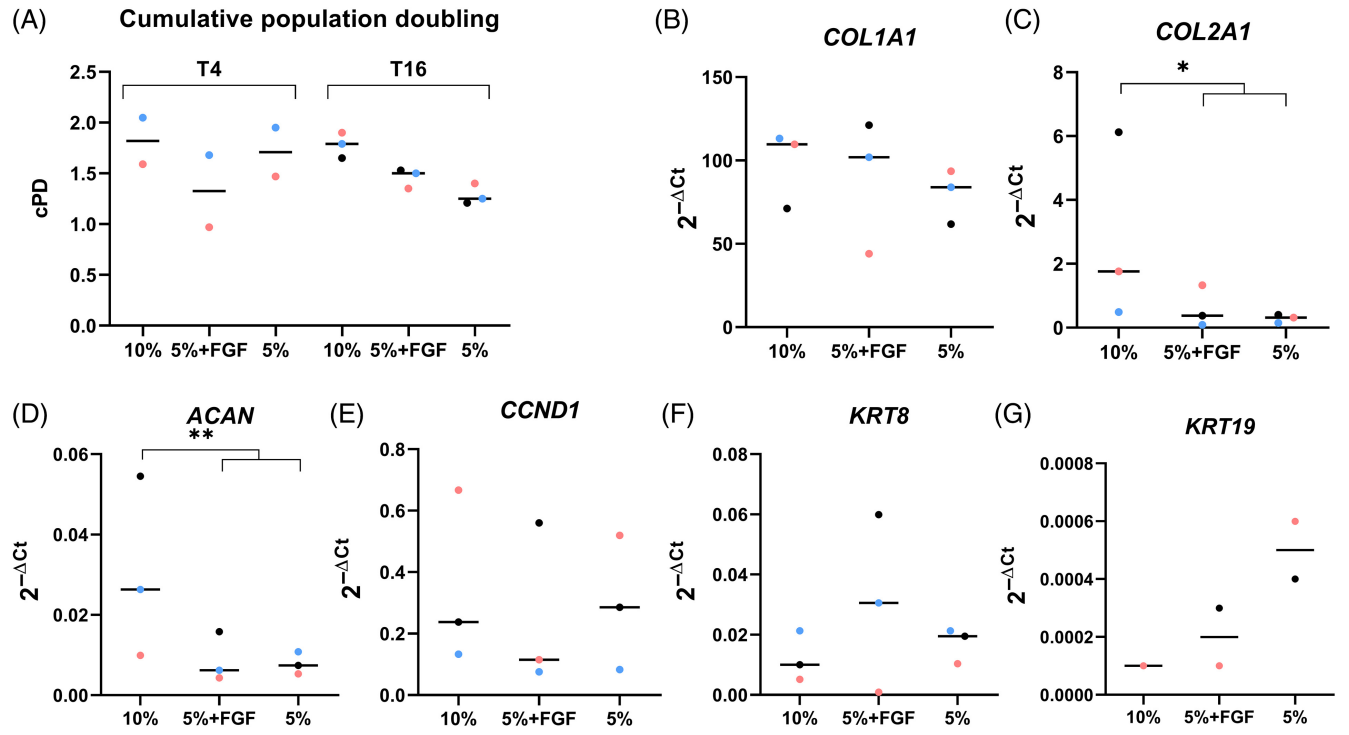

FIGURE 16 Effect of expansion on dog NP cell phenotype in reduced FCS media. Expansion of NP cells isolated from CD dog NP tissue. Cumulative population doubling from passage 0–3 (A). Gene expression for COL1A1 (B), COL2A1 (C), ACAN (D), CCND1 (E), KRT8 (F), and KRT19 (G) after passage 2 of expansion. Three different expansion conditions tested: 10% FCS, 5% FCS + 1 ng/ml FGF, and 5% FCS. One-way ANOVA with Tukey's post hoc tests for pairwise comparisons * $P < 0.05$. $n = 3$ dogs (A) in triplicate (B–G).

adhered to flasks coated with fibronectin in the absence of FCS these were very low numbers and failed to reach confluence within 1 month and could not be analyzed further. Plating human NP cells on Geltrex™ had no significant effect on either cell count or gene expression (Figure 19). The NP cells did not rapidly adapt to monolayer culture in the absence of FCS, as indicated by the limited cell numbers achieved at end of P0 and longer time it took to reach confluence prior to the first passage (Figure 19A), the addition of a thin layer of Geltrex™ did not significantly impact either the initial acclimation or subsequent passages. Media supplementation with FCS, however, had a substantial impact on the ability of human NP cells to survive and proliferate in monolayer culture (Figure 19A). For those cells that did survive in the serum-free media, the media supplementation did not have a significant effect on *ACAN*, *KRT18*, *COL1A1*, or *COL2A1* gene expression, although significant differences between individual media types were observed when the uncoated and Geltrex™-coated conditions were combined (Figure 19B–E).

3.9 | Alternatives to FCS in 3D culture

3.9.1 | Human

To determine the effects of FCS and alternatives on NP cell phenotype during 3D in vitro re-differentiation, human NP cells resuspended into alginate beads were cultured and supplemented with either FCS, ITS-X + Albumax or ITS-X + Albumax + L-proline. Following the 14-days culture period the DNA and GAG concentration of NP cells cultured in both alternative supplements (ITS-X, Albumax; ITS-X, Albumax, L-proline) was not significantly altered compared with FCS-supplemented NP cells, indicating that both alternative media supplements had no adverse effect on cell number and GAG production (Figure 20A,B). Also, the gene expression of NP cell phenotypic markers *KRT18*, *KRT19*, and *PAX1*, extracellular matrix markers *ACAN* and *COL2A1*, degradative enzymes *MMP3* and *ADAMTS4* and *IL1B* all remained unchanged across all treatment groups after 14 days re-differentiation (Figure 20C–J). Taken together, these results indicate that there were no adverse effects on cell viability and the phenotype of re-differentiated NP cells remained identical when cultured using alternative growth supplements compared with FCS. This demonstrates that either of the alternate growth supplementation methods can be utilized for NP cell culture once in 3D culture, without any change in phenotype during 3D re-differentiation.

3.10 | Freezing protocols

Protocols were submitted from 24 laboratories that routinely cryopreserve NP cells to liquid nitrogen. The majority used DMSO in their freezing recipe (Figure 21A). Some labs used ready to use freezing media with unknown components and only a few labs did not include DMSO for freezing NP cells and only used media and FCS of varying

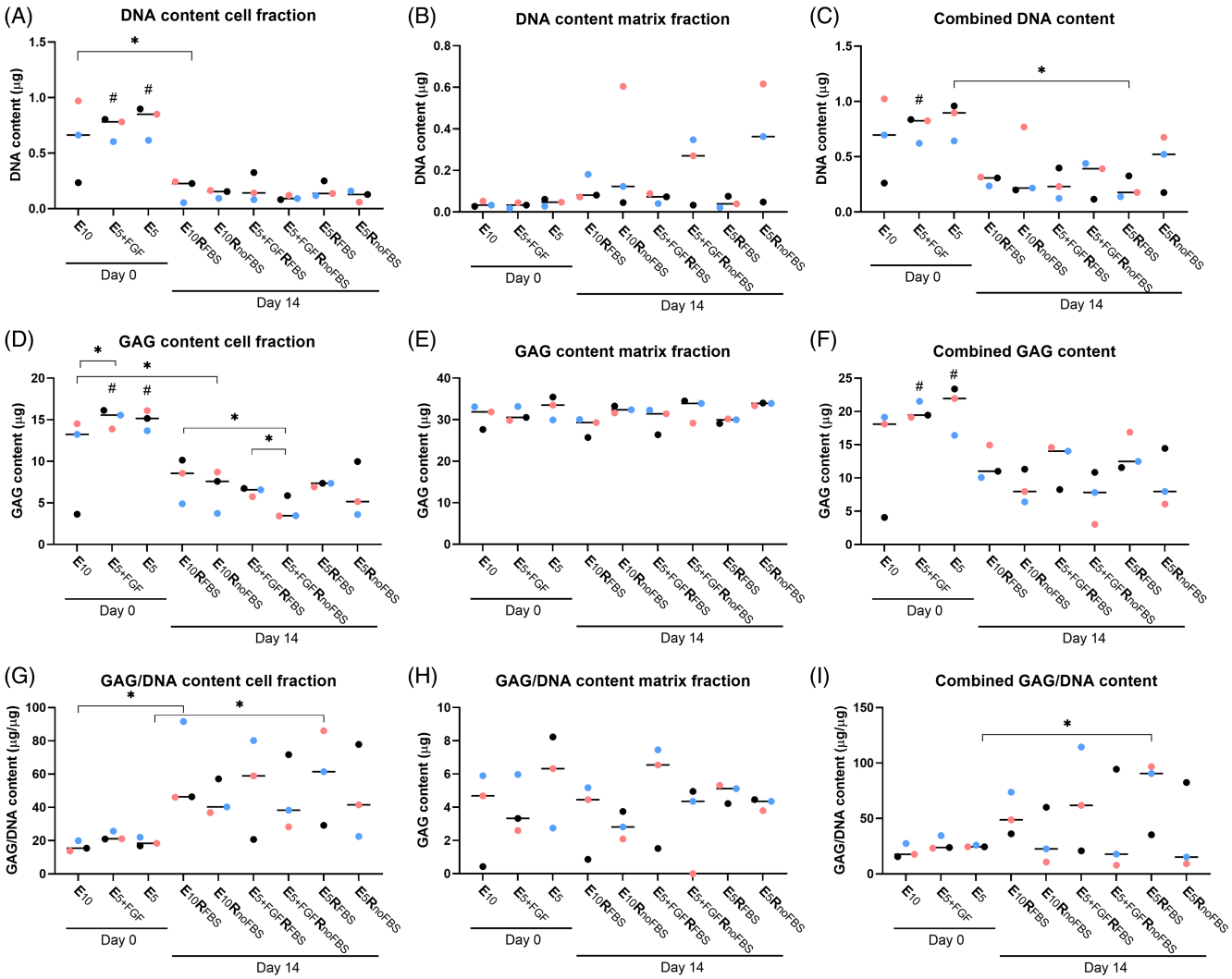

FIGURE 17 Re-differentiation of dog NP cells in reduced FCS media: DNA/GAG content: DNA content cell fraction (A), DNA content matrix fraction (B), combined DNA content (C), GAG content cell fraction (D), GAG content matrix fraction (E), combined GAG content (F), GAG/DNA content cell fraction (G), GAG/DNA content matrix fraction (H), and combined GAG/DNA content of dog NP cell alginate beads after redifferentiation in six different conditions (three different expansion media and two different redifferentiation media): (1) $E_{10\%}$ R_{FCS}, (2) $E_{10\%}$ R_{noFCS}, (3) $E_{5\%+FGF}$ R_{FCS}, (4) $E_{5\%+FGF}$ R_{noFCS}, (5) $E_{5\%}$ R_{FCS}, and (6) $E_{5\%}$ R_{noFCS}. $n = 3$ dogs. One-way ANOVA with Tukey's post hoc tests for pairwise comparisons * $P < 0.05$, #significantly different ($P < 0.05$) from day 14 samples in same expansion condition. $n = 3$ dogs in triplicate.

percentages (Figure 21B). Almost all protocols included freezing cells gradually by mixing initial number of cells with freezing media and reaching to −80°C slowly and then transfer to liquid nitrogen for longer freezing. During thawing, rapid thawing was reported by all groups, and most labs used a water bath at 37°C followed by centrifugation to remove DMSO prior to plating for expansion. While some groups report thawing the NP cells at room temperature.

4 | DISCUSSION

IDD is a major cause for BP, however, progression of research into fundamental cell biology and translational studies targeted at biological regeneration and repair are hampered by inconsistencies in methodology, making lab-to-lab comparisons difficult and multicenter studies problematic. In vitro cell culture is a widely utilized tool for investigating normal cellular biology, physiology, and pathophysiological processes together with investigating potential new therapeutic approaches. However, the lack of coherent standard operating procedures to enable comparison of experimental work is a major hurdle. The NP is an essential component of the IVD and is often considered as the initiation site for IDD. Thus, as a first step to develop consensus methodology for NP cells used for fundamental and translation research, this international, multicenter study focused on developing recommendations for NP cell isolation and expansion to reduce lab-to-lab variation and increase reproducibility across the spine field. Thirty-six research groups worldwide who routinely handle NP cells from various species contributed their protocols for in vitro culture of NP cells. Following expert review of methodology submitted together with considerations of prior publications, key commonalities were

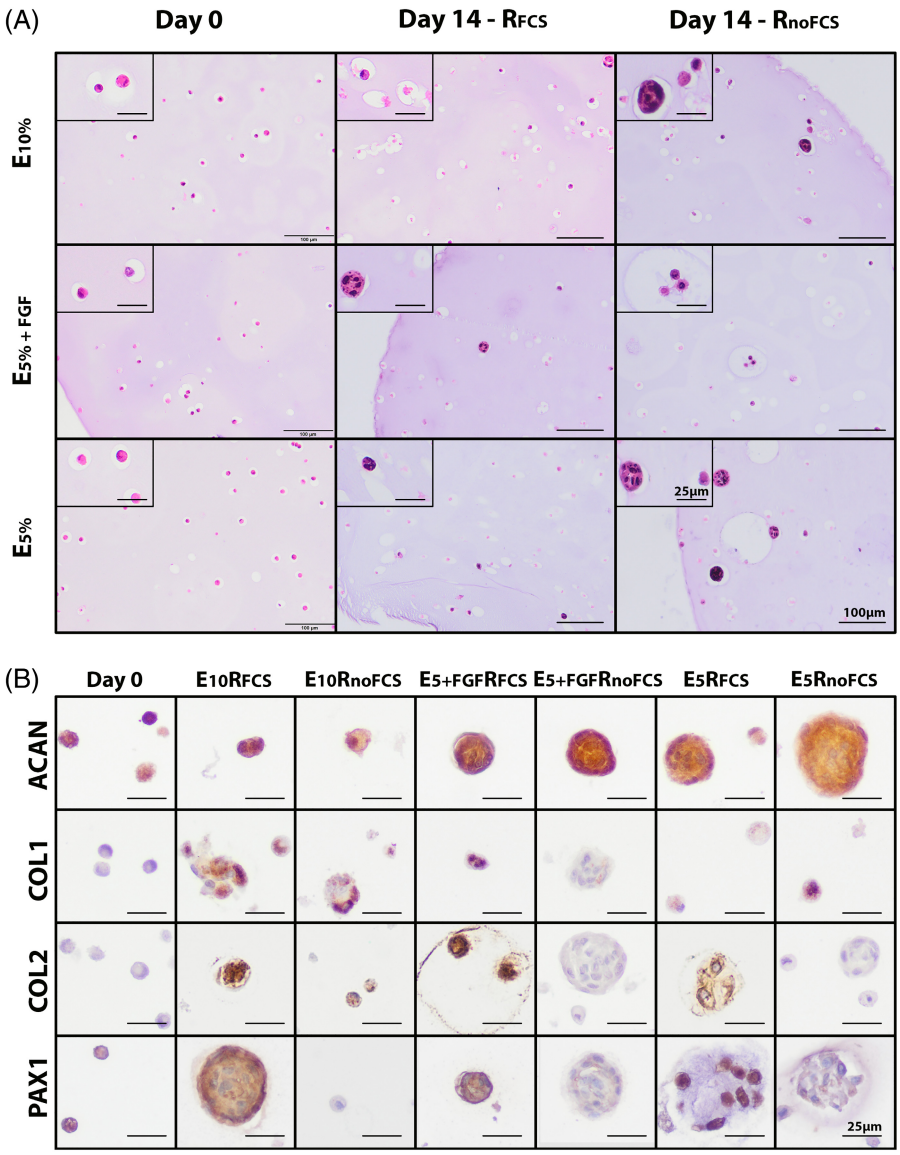

FIGURE 18 Re-differentiation of dog NP cells in reduced FCS media: Histology & IHC: Representative histological pictures of H&E-stained alginate beads (A) and immunohistochemical results for ACAN, COL1A1, COL2A1, and PAX1 after redifferentiation in six different conditions were (three different expansion media and two different redifferentiation media): (1) $E_{10\%}$ R_{FCS}, (2) $E_{10\%}$ R_{noFCS}, (3) $E_{5\%+FGF}$ R_{FCS}, (4) $E_{5\%+FGF}$ R_{noFCS}, (5) $E_{5\%}$ R_{FCS}, and (6) $E_{5\%}$ R_{noFCS}. $n = 3$ dogs.

identified for immediate recommendation, and differential factors were identified for experimental testing within the remit of this article. Furthermore, discussion of areas for future work identified which fell outside of the scope of this initial recommendation paper were agreed.

This article specifically aimed to test methodology and make recommendations for isolation and culture of rat, rabbit, pig, dog, cow, and human NP cells. With a specific focus on the small NP cells of the NP tissue, where NC isolation and phenotypic maintenance is the topic of a second article, where mouse tissues are also considered. Furthermore, features that can influence cell phenotype and proliferation during expansion of NP cells including basal media choice, tissue culture coatings, oxygen concentration, and alternatives to FCS were investigated, together with the influence of increasing passage number. We report recommendations for basic expansion of NP cells across species, highlighting the issues of loss of NP phenotype during expansion. Investigation of conditions which more closely mimic the physiological environment (e.g., O_2, pH, Osmolarity, glucose) were rarely reported in submitted protocols and considered outside the scope of this article. Due to issues of batch variability in FCS use and the problems this has on harmonization across labs we investigated potential alternatives to FCS during expansion in monolayer and re-differentiation of NP cells in 3D culture. This harmonization is hoped to foster the ultimate development of standardized NP cell culture methods in an international consensus statement to improve scientific comparability, utilization of funding and resources including number of animals (3R principle), and avoid repetitive experimental work in order to support the development of meaningful therapies for the treatment of IDD.[46,47]

4.1 | NP cell extraction

Groups from across the world contributed protocols for NP cell extraction including 25 groups for human NP tissue and 18 groups for animal tissues from a range of species (mouse, rat, rabbit, pig, dog, and

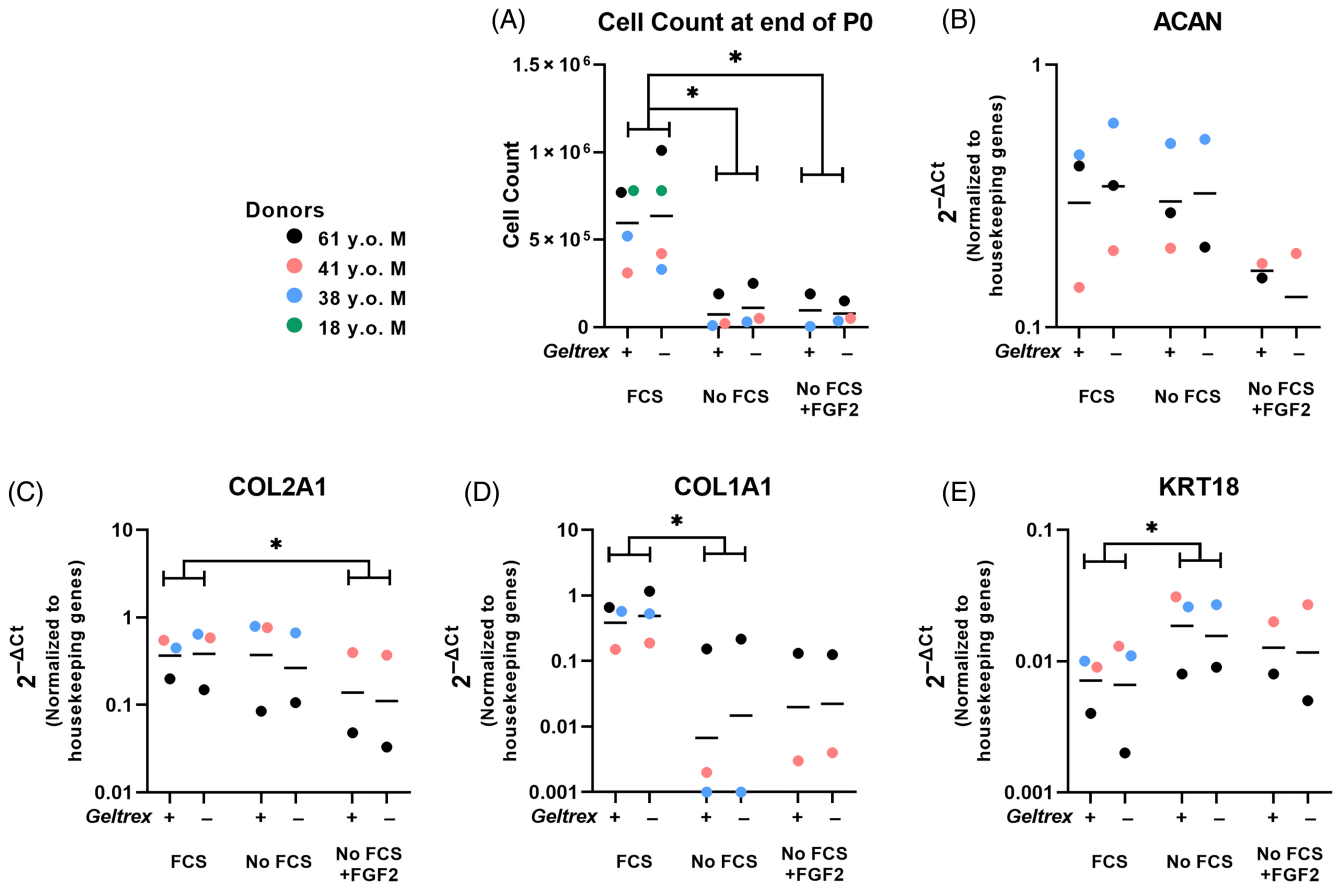

FIGURE 19 Effect of expansion on human NP cell population doubling and phenotype in FCS alternatives. The effects of different media supplements on human NP cells from recently deceased organ donors ($n = 4$) were investigated by culturing cells in high glucose DMEM with three media supplement combinations (10% FCS, "No FCS" containing Albumax/proline/ITS-X, or "No FCS + FGF-2" containing Albumax/proline/ITS-X/FGF-2) and on either Geltrex-coated or uncoated tissue culture plastic until end of P0 for assessment of cell count (A) and qRT-PCR (B–E). All qRT-PCR was normalized to GAPDH and RNA45S5. Two-way ANOVA with Tukey's post hoc tests *$P < 0.05$.

cow). Large variations in methodology were observed between protocols in terms of type, and concentrations of enzymes used, duration of incubation periods, and use and size of cell strainers. A greater consensus from submitted methodology was seen for use of cell strainer at 70 μm which was selected as a recommendation for this study. Survey data together with a review of the literature identified key protocols to investigate across the species, with cell yield, viability, and phenotype as output measures. Within the survey (and in publications), pronase and collagenase enzyme concentrations for cell isolation protocols were typically reported in terms of mg/ml or percentage weight per volume (%w/v) which were converted to mg/ml to enable analysis. While percentage weight per volume is based on physical characteristics that are easily determined in the laboratory, it is essential for the field to adopt units of activity (U/ml) to allow for more consistency in isolation protocols. It is important to note that depending on the manufacturer and batch of enzyme the unit of activity per gram can vary greatly (~10-fold), which was observed across lab sources in this study. In addition, the tissue mass to digestion solution volume ratio, will also influence the exposure of cells to enzymes and will ultimately affect both quality and cell yield; this was rarely reported in submitted protocols. We advocate consistent reporting in terms of units of enzyme and tissue mass to volume ratios of the digestion solution going forward.

In this work, we sought to develop a standardized protocol to maximize cell yield while minimizing adverse effects by investigating common features from published and submitted protocols within each species. Interestingly, although a wide range of methodologies were reported in submitted protocols and in the literature, experimental testing in this study has identified a number of similarities across species in final recommended extraction methodology, including: the use of pronase for rat, rabbit, pig, and chondrodystrophic dog; and combined enzyme treatment regimens rather than sequential digestions where tested (rat, rabbit, pig); similar collagenase concentrations (~60–100 U/ml) with collagenase type II recommended for most species with only rabbit where collagenase type P was investigated, and shorter digestion times across all species, although it is likely that collagenase type II would also be suitable for rabbit NP cells due to submitted methodology this was not investigated in the current study, of note the units reported for collagenase type P are reported by manufactures using differential units, thus conversion units are provided (Table 3).

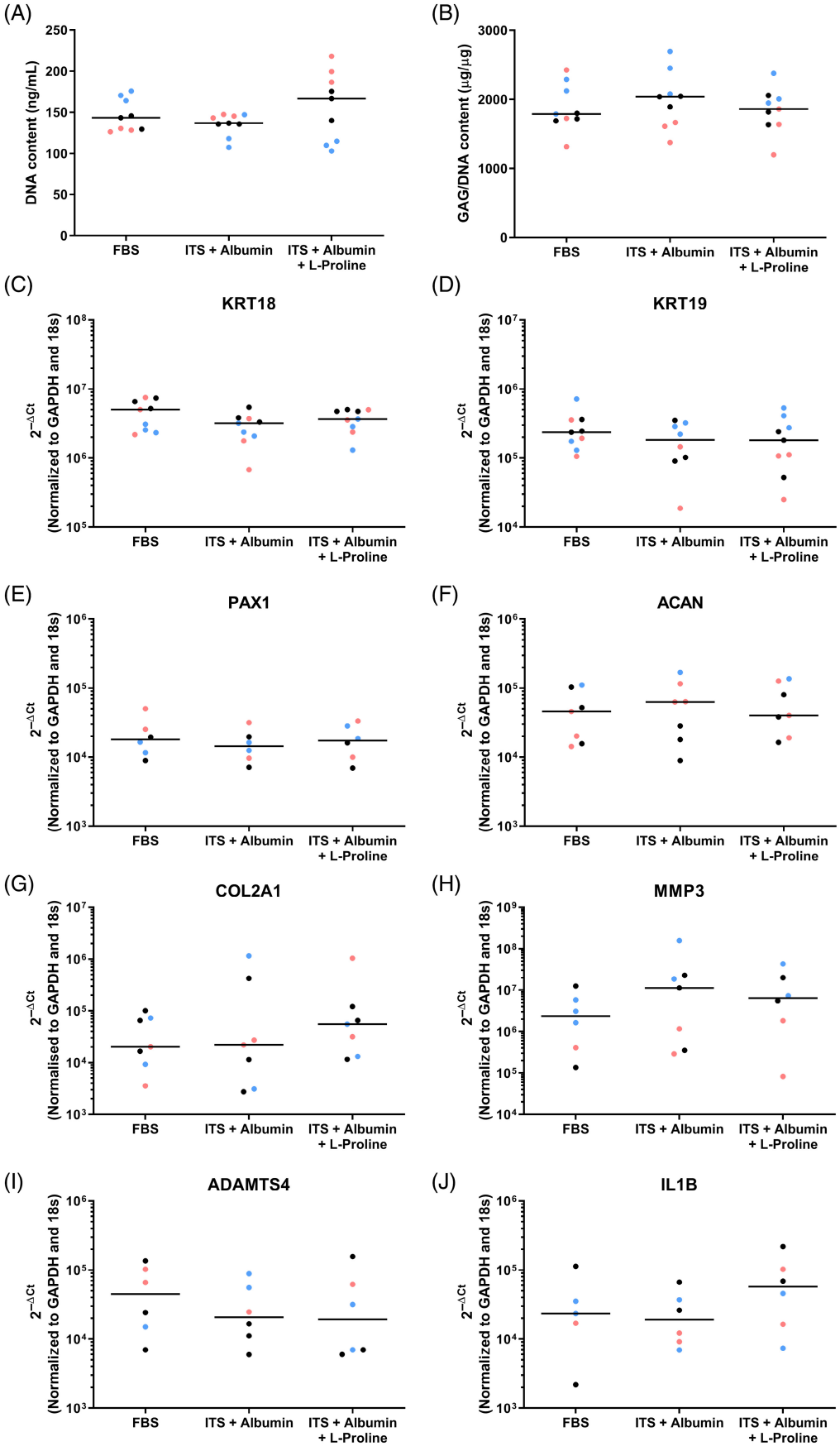

FIGURE 20 Legend on next page.

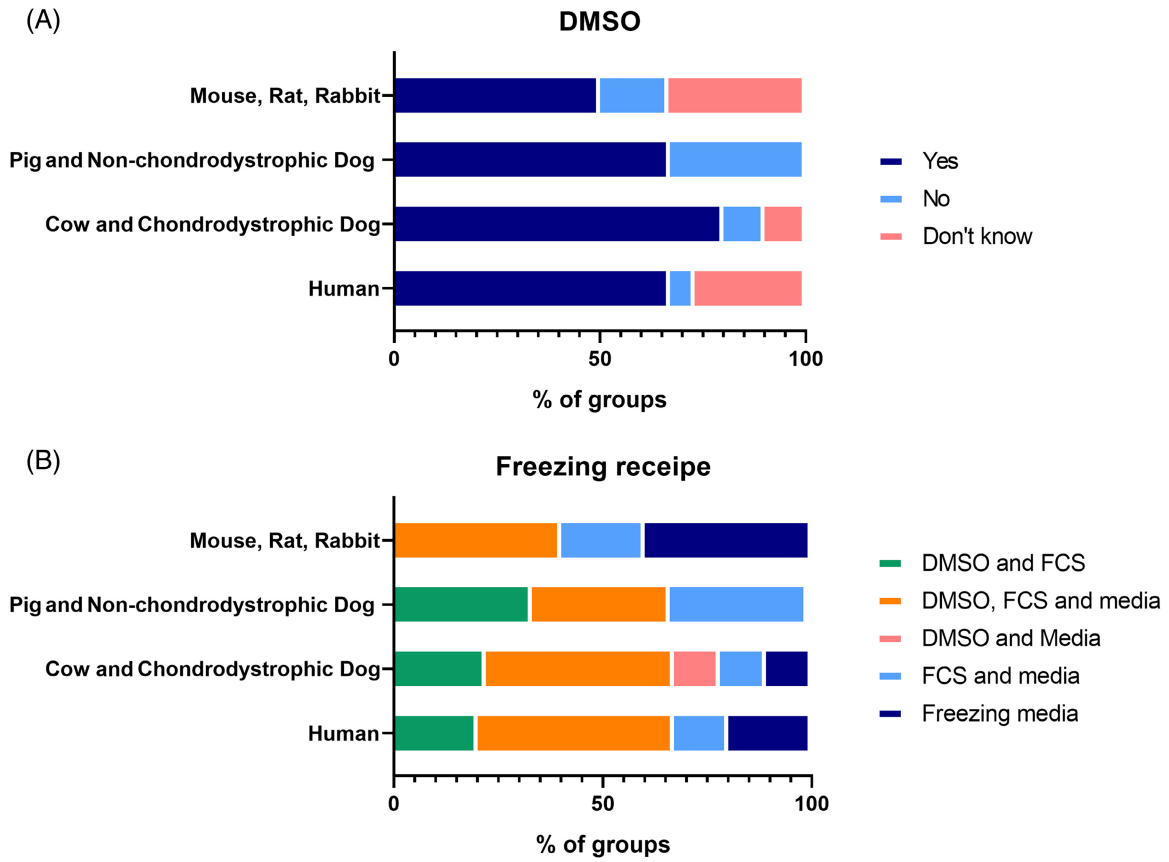

FIGURE 21 Freezing protocols. Demonstrated a range of freezing media and inclusion of DMSO, the majority of groups used a mixture of DMSO, FCS, and media to freeze cells.

Different protocols involving pronase and/or collagenase enzymatic digestions are found across the literature and in the submitted protocols, with double enzyme digestion methods reported more commonly for small animals. The utilization of a double enzyme digestion protocol was investigated in this study for rats, rabbits, and pig discs, where the NP cell mass is compact, involving extensive cell–cell interaction and clusters that cannot be effectively released by collagenase.[39,48] The inclusion of pronase causes partial ECM loosening[39,48] as pronase contains several non-specific proteases from *Streptomyces griseus* which digest proteins down to single amino acids.[49,50] However, in tissues where NP cells are more dispersed in the ECM such as cow and human NP tissues, pronase has previously been shown not to be required,[39] thus for cow and human discs pronase inclusion was not investigated further here. For dog NP cell extraction, the inclusion or exclusion of a pronase treatment was investigated. In a similar manner seen previously for cow and human NP cell extraction, pronase was not essential to yield NP cell release from dog discs, although small but non-significant increases in cell yield and viability were observed in the presence of pronase and thus, we recommend its inclusion for dog NP digestion (Table 3). However, as pronase has also been shown to have a damaging effect on cell properties of chondrocytes,[51] neurons,[52] Kupffer cells,[53] and lymphocytes,[54] where it is not required for cell extraction, such as cow and human discs, we recommend exclusion (Table 3). Most extraction methodology across species showed a high degree of cell viability and similar mRNA expression patterns, suggesting negligible effects on cell phenotype or damage as a factor of the protocol used. Within rat, rabbit, and porcine NP cells, combined exposure to pronase and collagenase

FIGURE 20 Re-differentiation of human NP cells in FCS replacement media. After resuspending human NP cells ($n = 3$) into alginate beads 2 weeks in a 3D environment. During the 2-week re-differentiation period alginate beads ($n = 24$ per patient) were cultured in 1 of 3 different media to test the effect of FCS and alternatives on the re-differentiation process. The basal composition of each media treatment was identical (low glucose DMEM), with the addition of (1) 10% (w/v) FCS, (2) 1% (w/v) ITS-X, or (3) 1% (w/v) ITS-X, 40 µg/ml L-proline. Human NP cells extracted from 3 individual patients were used in each separate experiment. Alginate beads were cultured for 2 weeks at 5% O_2 to allow human NP cells to re-differentiate into an in vivo like phenotype. Beads were harvested after 2 weeks, dissolved and cells were taken for (A) DNA and (B) glycosaminoglycan (GAG) content (normalized to DNA content) analysis, and gene expression analysis of (C) KRT18, (D) KRT19, (E) PAX1, (F) ACAN, (G) COL2A1, (H) MMP3, (I) ADAMTS4, and (J) IL1B. One-way ANOVA with Tukey's post hoc tests were performed to investigate statistical differences. No statistically significant differences were observed between the three different media conditions.

TABLE 3 Recommended protocols for NP cell extraction

Animal model	Enzymes and concentration	Digestion media	Tissue: volume ratio	Digestion duration	Cell strainer	Anticipated cell yield
Rat	Pronase (14 U/ml) Collagenase type II (100 U/ml)	hgDMEM, 1% P/S, 110 mg/L pyruvate	Combine NP from 6 IVDs: 1 ml per disc	6 h @ 37°C	70 μm	3–4 × 10^4 cells/disc
Rabbit	Pronase (62 U/ml) Collagenase P (0.4 U/ml[a]) (Collagenase type II likely to be sufficient but currently untested)	hgDMEM, 1%P/S	Combine NP from 6–10 IVDs: ~2.5 ml per disc	2 h @ 37°C	70 μm	~1.5 × 10^5 cells/disc
Pig	Pronase (14 U/ml) Collagenase type II (64 U/ml)	hgDMEM +5% FCS	30 ml/g tissue	4 h @ 37°C	70 μm	~2 × 10^6 cells/g
Dog	Pronase (5 U/ml) Collagenase type II (64 U/ml)	hgDMEM	20 ml/g tissue	Pronase 30 min @ 37°C Collagenase 4 h @ 37°C	70 μm	~5 × 10^6 cells/g
Cow	Collagenase type II (64 U/ml)	hgDMEM, 1% P/S	20 ml/g tissue	4 h @ 37°C	70 μm	~2 × 10^6 cells/g
Human	Collagenase type II (64 U/ml)	hgDMEM, 1% P/S	20 ml/g tissue	4 h @ 37°C	70 μm	~1 × 10^6 cells/g

Note: Ideally enzymes should be made fresh for use, however enzymes can be prepared as stock solution if needed. For this, prepare a 5× stock in DMEM and store in −20°C as small one-use aliquots. Do not refreeze once defrosted. Storing at lower concentrations may lead to loss in activity over time. Prepare working solution fresh and use immediately. Mouse is the subject of a companion paper on Notochordal Cell extractions.
Abbreviations: FCS, fetal calf serum; IVD, intervertebral disc; NP, nucleus pulposus; P/S, penicillin/streptomycin.
[a]Note reported units for Collagenase type P are given in Wunsch Units, conversion to Mandl units to match Collagenase type II = 400 U/ml (Mandl).

promoted the highest cell yields as opposed to sequential digestion (Table 3).

Duration of enzyme treatments was also seen to vary substantially between submitted protocols from <2 h to overnight digestions reported, although overnight incubations were listed for all species, shorter incubation periods of <2 h were more common for mouse, rat, and rabbit discs, 2–4 h for pig and some dog discs, while a full spectrum were reported for human discs. The greater variability seen for human discs may be reflective of the degenerative state, where severely degenerated human NP tissues tend to be more fibrous and require longer incubation times compared with gelatinous NP tissues of healthy IVDs. However, increased digestion time has been previously shown within nasal chondrocytes to decrease viability, proliferation, and extracellular matrix-forming capacity (GAG and collagen).[55] Similarly in the current study, limited increases in cell yield were observed when NP tissues were extracted overnight (rabbit, pig, dog, cow, and human) compared with shorter 2 h (rabbit) and 4 h (pig, dog, cow, human) incubations, however, decreased gene expression for key NP cell phenotypic markers was observed with longer collagenase treatments. Thus, we recommend utilizing shorter incubation periods to prevent loss of NP phenotype during extraction (Table 3). While this study did not investigate the effect of age on cell extraction methodology, given the commonality in optimized methodology across species including older human samples, it is likely that the methods recommended should be applicable to all ages.

Interestingly, where extraction methodology for rat tissues was investigated across three sites, only one research group had sufficiently high cell yields to accurately perform RT-qPCR. This highlights the technical challenges in digesting small volumes of tissue from the discs of rodents, and the need for training, collaboration, and sharing of knowledge among the research community, demonstrating the importance of exchange of personnel, and travel grants for young researchers. Extraction of RNA from NP tissue is technically challenging given the low cell density in a proteoglycan-rich extracellular matrix. Furthermore, despite all groups using the same active unit concentrations of enzymes, there may have been differences in the quality and purity of enzymes from different manufacturers.[39] Overall, we note that when working with NP tissues from smaller animals (mice, rats, and rabbits) it is important to obtain adequate tissue mass to effectively isolate enough cell numbers. While cell isolation from individual discs is possible for techniques such as RNA extraction[35] and single cell sequencing,[56] pooling together several discs from one animal increases feasibility. However, particularly for rodents and rabbits it may be technically difficult to obtain enough tissue from a single biological replicate for experiments requiring high cell numbers for forming 3D culture systems with cell yields per disc of 3–4 × 10^4 cells from rats and 15 × 10^4 cells from rabbit discs. Thus, discs from across multiple animals may need to be combined. Furthermore, due to the relatively low volume of NP tissue in each rat disc, significant attention to detail and care should be employed during dissections, as any amount of tissue loss may become significant. It is recommended to expose the NP by making a transverse incision as close to the endplate as possible to diminish any shearing events caused by running the blade through the NP, which may result in the loss of NP tissue to the blade or surrounding tissues. Leaving the NP tissue as intact as possible for small animal tissues prior to digestion can further improve cell yields as it decreases tissue loss due to increased handling.

4.2 | Expansion and differentiation

Culture media compositions were collected from 36 groups worldwide, and while most groups reported utilizing hgDMEM, five different basal medias were reported. Here, the most common basal medias were tested across several species and limited differences were seen in phenotype and population doubling behavior between hgDMEM, hgDMEM/F12, or hgHamsF12. Since increased NP cell markers were seen in human NP cells cultured in hgDMEM as opposed to DMEM/F12 thus we recommend standardizing future cultures using hgDMEM (Table 4). Most groups included antibiotics and fungizone, as a preventative measure for primary cells, however, the use of antibiotics has been shown to affect the cell activity and functionality and thus should be considered.[57,58] As such, while we have included these in our recommended media composition for expansion (Table 4), their use should be kept to a minimum following extraction and preferentially removed during experimental procedures.

Ascorbic acid was utilized in most protocols reported for cow, chondrodystrophic dog, and human NP cells. Very few groups culturing mouse, rat, rabbit, pig, or non-chondrodystrophic dog NP cells used ascorbic acid, although many of these groups utilized αMEM which already contains L-ascorbic acid. As ascorbic acid is essential for collagen deposition,[59,60] a key feature of the NP cell phenotype, this was included in all cultures in this study and included in our recommended culture media regardless of the species (Table 4). We also specifically recommended the use of L-ascorbic acid 2-phosphate which is more stable in culture media,[61] if L-AA is utilized then this should be added fresh at each media change, thus the use of media already containing L-AA such as αMEM is not suitable. It should be noted that ascorbate can also generate H_2O_2 in culture media which can cause cytotoxicity,[62] however its effects can be reduced by the antioxidant activity of pyruvate in the culture media.[63,64] Additionally, pyruvate is applied to improve metabolism of glucose acting as an intermediate in the glycolytic pathway and thus can improve cell survival in vitro particularly under high glucose conditions[65] such as those used for NP cell expansion. Most protocols submitted for NP cell culture reported using pyruvate and given the benefits for cell culture we recommend either the use of media already containing this (Table 4) or adding it as a supplement.

The addition of growth factors was reported by very few groups during both expansion and differentiation and included either FGF-2, PDGF, or TGF with FGF-2 being the most commonly used. FGF-2 is often-used as a mitogen to support expansion of cells[66] and has been shown to maintain cells in a stem state and is often used for MSC culture to maintain their chondrogenic capacity.[67] However, while it has been reported to enhance ECM synthesis in some contexts, it has also been shown to suppress ECM synthesis in other studies, which may be cell type dependent.[68–70] The inclusion of FGF-2 within the current study with dog or human NP cells displayed no advantage or increase in cumulative population doubling, while decreased NP cell marker expression was observed. Thus, we do not recommend its inclusion for NP cell expansion. All groups reported using FCS within their culture media for monolayer culture with concentrations ranging from 1% to 20% with 10% being the most common. However, as FCS is an ill-defined cocktail[71] and suffers from substantial batch to batch variability[72] the inclusion of FCS within culture media is problematic for consistency between groups. FCS is utilized to provide a cocktail of ECM components, growth factors, hormones, minerals, and proteins which cells require to adhere to tissue culture plastic and proliferate successfully.

Alternatives to FCS in culture media include defined media substitutes such as ITS (Insulin, Transferrin, Selenium), combined with Albumin, and coating tissue culture plastic with ECM components such as fibronectin, laminin, Matrigel, or Geltrex or gel systems such as gelatin.[72,73] Here, several strategies were investigated to determine whether NP cells could be expanded in monolayer culture without the need for FCS, which would improve consistency between labs. However, neither dog nor human NP cells were able to adhere well to tissue culture plastic in the absence of FCS even when culture plates were coated with laminin, fibronectin, or Geltrex™. Dog NP cells were unable to be analyzed in the absence of FCS, and while some human NP cells adhered to uncoated, fibronectin or Geltrex™ coated plates in the absence of FCS, very limited cells adhered and they failed to reach confluence even after 1 month in culture, with coatings showing no advantage over tissue culture plastic. As such, despite the limitations of FCS, our current recommendation is that FCS is maintained in expansion media (Table 4), but batch testing needs to be performed and if working in consortia projects, where it is essential to compare data between sites, shared FCS batches are preferred. Furthermore, reduced FCS concentration was investigated for dog NP cells and, although ECM-related gene expression was decreased when lowering the concentration of FCS from 10% to 5% during expansion, the dog

TABLE 4 Recommended media for NP cell expansion (All species)

Product	Concentration	Suggested Supplier and product code
DMEM high glucose, GLUTAMAX, with pyruvate	As supplied	Gibco: 10569010 HG
Penicillin/streptomycin	1% w/v penicillin, streptomycin (optional)	Gibco: 15070-063
Amphotericin B	25 µg/ml (optional)	Sigma: A2942
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	50 µg/ml	Sigma: A8960
Fetal calf serum	10% v/v	Batch and supplier to be determined—ensure batch testing completed

Note: Antibiotics and anti-fungal are commonly used esp. following extraction but optional. Standard tissue culture plastic is recommended as coatings with fibronectin, Gelatin, Geltrex, and laminin showed no advantage in the investigations undertaken.

NPCs were still viable and proliferating. The addition of FCS during redifferentiation resulted in increased gene and protein expression of COL2A1 and a higher GAG content. Together with the increased PAX1 immunopositivity in the presence of FCS during redifferentiation indicates that FCS improves the normal phenotype and regenerative capacity of the dog NP cells. This in contrast with Arkesteijn et al. (https://pure.tue.nl/ws/files/25509102/20160628_Arkesteijn.pdf) where dog NC phenotype was retained better in 3D culture in serum-free media; however, the inhibition response seen in this study by FCS could be explained by the difference in cell type or FCS batch.

During expansion in culture, it is well known that cells isolated from the IVD de-differentiate and become fibroblast-like both in appearance and matrix synthesis, with decreased expression of key NP marker genes and matrix proteins such as collagen type II and aggrecan and induction of collagen type I.[41,74–76] Here, loss of NP cell phenotype during expansion from all species investigated was also observed, with decreased NP cell phenotype over short culture periods. With a switch in matrix expression from collagen type II and aggrecan to collagen type I rapidly in culture with significant switches seen after passage 2, while other key NP markers such as KRTs and brachyury were lost even earlier in culture during the first passage. Expansion durations should also be limited to prevent induction of cellular senescence in cultured NP cells which, for non-degenerate human NP cells, show decreased population doubling capacity around passage 10 to 11.[77] Cells derived from degenerate human discs display decreased population doubling capacity and undergo senescence around passage 4 to 5.[77] Thus, it is essential to limit passage number to prevent induction of senescence and loss of phenotype and transfer cells from 2D culture into a 3D culture system which can drive re-differentiation of NP cells, we would recommend utilizing cells preferentially by passage 2 or 3 as even at these low passages changes in phenotype were observed in monolayer culture.

Culture of NP cells in 3D culture systems such as pellet, micromass, alginate, agarose, and collagen scaffolds have been reported previously to support re-differentiation of NP cells to the in vivo phenotype, with alginate culture reported most commonly in the literature[12,78] and within the submitted protocols. Thus, alginate was utilized within this study to investigate culture media for re-differentiation for FCS alternatives. However, while alginate culture supported human NP cells well, dog NP cells showed decreased DNA content from day 0 to day 14. Although the cells which remained in alginate cultures showed cluster formation and excellent matrix deposition and NP phenotypic expression, with similar cell density visible on histological slices. Thus, the decreased DNA content measured may be due to reduced cell numbers in culture due to differential metabolic profiles of dog and human NP cells, or reduced extraction efficiency following alginate culture. 3D cultures in this study were performed under 5% O_2 to mimic the in vivo environment and support re-differentiation. When moving from monolayer cultures to 3D cultures 25% of submitted protocols reported the use of culture media without FCS. Testing alternatives to FCS using human NP cells in alginate culture under 5% O_2 and lgDMEM, demonstrated no difference between cultures with FCS and the alternatives. As cells in 3D culture do not require the cell adhesion substrates provided by FCS which was essential in expansion media,[79] we would recommend shifting to FCS free media during re-differentiation, and the use of 3D culture system for experimental procedures (Table 5). Thus, supporting appropriate NP cell phenotype, while also reducing lab-to-lab variability which can be induced from FCS batches.

TABLE 5 Recommended culture and media for FCS free re-differentiation human NP cells in 3D alginate culture

Product	Concentration	Suggested supplier and product code
Low glucose DMEM (1 g/L glucose + pyruvate)	Made up as per manufacturers guidelines	Gibco: 31600-083
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	50 µg/ml	Sigma: A8960
L-glutamine	1% v/v	Gibco: 25030-024
ITS-X	1% v/v	Gibco: 51500-056
L-proline	40 µg/ml	Sigma: P5607
Albumax	1.25 mg/ml	Gibco: 11020-021

Note: Alginate culture is not recommended for dog NP cells, and other species should be tested to confirm phenotypic maintenance.

4.3 | Cryopreservation

The majority of protocols submitted for cryopreservation of NP cells utilized DMSO together with FCS within their freezing media. DMSO is commonly applied during cryopreservation to prevent the formation of intracellular water crystals which would be toxic to the cells.[80] Furthermore, DMSO increases the permeability of the cell membrane enabling free flow of water through the membrane preventing osmotic stress which can occur during freezing.[80] Cell death during freezing is further prevented by the gradual freezing of cells,[81] which was reported in the majority of protocols submitted, which described slow freezing to −80°C, followed by transfer to liquid nitrogen for longer term cryopreservation which also helps to prevent ice crystal formation.[82] During thawing, rapid thawing was reported by all groups, rapid thawing also decreases the time defrosted cells are in contact with DMSO which can be toxic, and thus thawed cells should be placed in complete media and DMSO removed rapidly following defrosting. As methodology reported was similar in general principles and following expert review of protocols, we recommend the resuspending 1×10^6 cells/ml in 10% DMSO/90% FCS v/v; which should be frozen utilizing a slow freezing method at −80°C (e.g., with the use of a Mr Frosty™ or insulated packaging) for a minimum of 4–16 h max prior to transfer to liquid nitrogen, upon defrosting vials should be

TABLE 6 Checklist for reporting cell culture extraction and expansion methodology

Cell Source	Extraction	Culture Media	Expansion	Re-differentiation
Disc region	Weight of tissue/number of discs used	Basal media	Culture method—2D, 3D	Culture method utilized
Disc level	Enzymes used including type, source and Cat Number	Glucose concentration	Tissue culture plastic/coatings used. Full details	Seeding density
Species and strain/breed (if animal)	Enzyme concentration in Units	Glutamine source	Seeding density/confluence level at passage	Duration required to induce re-differentiation
Degeneration state (classified using histological grading on matched sample) using JOR spine consensus histology grading series	Digestion duration, rotation speeds and whether enzymes combined or sequentially digested	Inclusion of pyruvate	Passage number utilized, preferably population doubling	Culture media and conditions as per media and expansion
	Digestion media utilized	Antibiotics and fungicides used	O_2 and CO_2 conditions	
	Cell strainer size utilized	Ascorbic acid type and concentration and refresh period		
		FCS used if so company and batch		
		Further supplements full details		

thawed rapidly and transferred to complete media while small ice still remains.

4.4 | Future directions

This study identified recommendations for standardization of extraction methodology across species (Table 3), a basal culture media for cellular expansion across all species (Table 4) and re-differentiation of NP cells (Table 5). However, the culture conditions described here do not consider the native disc environment, apart from partial consideration during re-differentiation where lgDMEM and culture under 5% O_2 was deployed. However, the native disc environment displays differential osmolarity, pH, glucose, and O_2 levels which will also differ between species and thus may need to be considered where the native tissue niche is important. The tissue niche also changes considerably during degeneration and thus when investigating cellular pathogenesis and future therapeutic approaches researchers may wish to modulate culture conditions to mimic more closely the in vivo tissue niche such as those proposed previously.[12]

The harmonization of NP cell culture extraction, expansion, and re-differentiation methodology performed in this article, however, is just the first step in developing a complete in vitro toolkit for IVD researchers. This model utilizing an international collaborative approach can now be utilized to develop harmonized methodology for NC, AF, CEP cells, tissue culture explants and whole organ cultures enabling comparability and transparency in discovery and translational science. To ensure appropriate reporting of cell culture methodology we have provided a checklist which we recommend journals utilize to ensure complete reporting of cell culture methods (Table 6).

4.5 | Outlook

The global burden of BP due to IDD as well as the consecutive costs on health care systems will further increase dramatically unless improvements to treatment strategies or prevention are developed. Although general interest, funding, and research on IVD regeneration have increased significantly within the last 20 years, the additional scientific volume has not yet translated into meaningful improvements in the treatment of patients having IDD. The unmet need for better care with causative therapies for patients having IDD can only be mastered in a multidisciplinary team approach including scientists, engineers, surgeons, and industry. Substantial governmental funding in addition to private investments and/or industry for enhanced international research co-operations and communication will be necessary to translate preclinical approaches more rapidly into meaningful discoveries that ultimately find their way into routine clinical practice. As experts in the field representing the ORS Spine Section and beyond, it is our duty to utilize public funding as efficiently as possible, precisely define pathologies (such as IDD), standardize models of disease, and finally conduct impactful and relevant research that translates into clinical practice. This work aims to contribute to this mission.

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CONFLICT OF INTEREST

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

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ENDNOTE

* Consensus terminology is lacking for defining the cells residing within the NP from development to adulthood. In this protocol manuscript, the cells residing in the embryonic notochordas embryonic notochord cells (eNCs). Once the IVD has developed, the notochord-derived large, vacuolated cells found in the prenatal fetal and postnatal (juvenile) NP are termed here as notochordal cells (NCs). Once these cells transition to smaller non-vacuolated chondrocyte-like cells they are termed nucleus pulposus cells (NP cells).

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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