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RNA *in situ* hybridization characterization of non-enzymatic derived bovine intervertebral disc cell lineages suggests progenitor cell potential

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ABSTRACT

Degeneration of the intervertebral disc (IVD) is a meritorious target for therapeutic cell based regenerative medicine approaches, however, controversy over what defines the precise identity of mature IVD cells and lack of single cell based quality control measures is of concern. *Bos taurus* and human IVDs are histologically more similar than is *Mus musculus*. The mature bovine IVD is well suited as model system for technology development to be translated into therapeutic cell based regenerative medicine applications. We present a reproducible non-enzymatic protocol to isolate cell progenitor populations of three distinct areas of the mature bovine IVD. Bovine specific RNA probes were validated *in situ* and employed to assess fate changes, heterogeneity, stem cell characteristics and differentiation potential of the cultures. Quality control measures with single cell resolution like RNA *in situ* hybridization to assess culture heterogeneity (PISH) followed by optimization of culture conditions could be translated to human IVD cell culture to increase the safety of cell based regenerative medicine.

1. Introduction

With an increase in average life expectancy, modern society faces the challenges and burdens of age related medical conditions like intervertebral disc (IVD) degeneration. Regenerative medicine (RM) seeks to address these problems via cell-based approaches, replacing ailing cells, tissues or entire organs. Autologous mesenchymal stem cells (MSC) are of increasing interest to the field (Risbud et al., 2004b; Sakai and Andersson, 2015) circumventing ethical issues associated with embryonic stem (ES) cells and less likely associated with tumorigenicity than induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). Several *in vivo* niches for MSCs have been identified over the years, with bone marrow MSCs likely being one of the most studied ones (Bara et al., 2014).

Like for any organ targeted for RM, the objective to direct patient-specific cells towards an IVD fate is appealing, yet for safety reasons, there is a desperate need for single cell resolution quality control measures, especially in the wake of reported malpractice associated with an increase in stem cell clinics and a flourishing stem cell tourism market (Berkowitz et al., 2016). Identification of characteristic IVD lineage markers is important, yet none has been conclusively identified to date (Lv et al., 2014). Commonly RT-PCR or microarray expression profiling on pooled cells is applied to identify IVD cells *in situ* or *in vitro* (Arufe et al., 2010; Lee et al., 2015; Liu et al., 2011; Lv et al., 2014; Minogue et al., 2010; Thorpe et al., 2016). This pooling could mask heterogeneous cell populations and cause misidentification of cells.

The IVD is suited for RM approaches, as ailing discs might be identified long before an acute medical problem arises, allowing to generate autologous cells in culture. IVDs are the largest non-vascularized structures in our body (Moore, 2006), acting as semi-movable joints between the vertebral bodies (VB) providing flexibility and load transmission in the spine (Michalek and Iatridis, 2012; Moore, 2006). The mature IVD consists of minimally four distinct tissue types: 1) The shock absorbing nucleus pulposus (NP); 2) the ringed exteriorly positioned annulus fibrosus (AF); 3) the transition zone (TZ) which is positioned between the NP and the AF and; 4) the endplates which sandwich the NP, TZ and AF to adjacent VBs (Pooni et al., 1986). Anatomically, mature IVDs appear as simple organs, but patterning events during embryogenesis resulting in different mesoderm derived cell lineages and structures are complex (Bara et al., 2014; Choi et al., 2008; Choi and Harfe, 2011; Choi et al., 2012; Maier and Harfe, 2011; Maier et al., 2013; Risbud et al., 2010; Sivakamasundari and Lufkin, 2012; Smith et al., 2011)

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and still not fully understood. Amenable to genetic manipulation, the mouse has long served as a model organism for studying vertebral column (VC) development (Chatterjee et al., 2013, 2012, 2014; Choi et al., 2008; Choi and Harfe, 2011; Choi et al., 2012; Kraus et al., 2014a; Sivakamasundari et al., 2012, 2013; Tribioli and Lufkin, 1999; Yap et al., 2011). Elegant *in vivo* studies have demonstrated that the notochord, a transient embryonic signaling center, gives rise to cells of the murine NP (Choi et al., 2012), while sclerotome cells contribute to the forming AF and VB. Lack of consensus over NP cell composition (Thorpe et al., 2016) in other species, including human, where the adult NP appears comprised of different cell types (Alini et al., 2008; Chelberg et al., 1995; Moriguchi et al., 2016; Pattappa et al., 2012) requires refinement. Recently different cell populations were isolated from the human and bovine adult NP using a variation of enzymatic tissue breakdown methods (Lee et al., 2015; Molinos et al., 2015; Turner et al., 2016) including multiple immortalized subpopulations of the human NP (van den Akker et al., 2014).

For ethical reasons, animal models are often employed to understand the biology of a target organ and they have in numerous cases served well (Alini et al., 2008; Kraus et al., 2014a; Vrana et al., 2014). We point to the histological difference between caudal adult rodent, porcine and bovine NPs, with the adult bovine NP more closely resembling human tissue, adding value to the bovine model system in understanding IVD development and composition. Thus having chosen *Bos taurus* as working model, we isolated cells of the AF, TZ and NP areas from several donors. We used a simple non-enzymatic method for cell isolation and characterized the individually derived cell lines at single cell resolution by plate RNA *in situ* hybridization (PISH) (Kraus et al., 2015) and histology. PISH indicated that all AF, TZ and NP cell lines derived from the mature bovine IVD represent heterogeneous cell populations, similar to our *in vivo* observations using RNA *in situ* hybridization on tissue sections (SISH) of the adult bovine IVD. Further analysis suggested that these cells are progenitor cells with a potential to generate extracellular matrix (ECM), and distinctly different from MSCs. An anticipated change in gene expression profile (Alini et al., 2008; Bara et al., 2014; Sakai and Grad, 2015) was most notably through changes in *Col1a1* expression *in vitro*, which *in vivo* was most prominent in the AF. This change in cell identity upon transferring IVD cells from their natural low nutrient microenvironment to monolayer cell culture reinforces the need for optimized culture conditions (van den Akker et al., 2014) and quality control measures with single cell resolution, especially, if cells were to be used for RM purposes.

2. Materials and methods

2.1. Tissue collection and processing for histology

Bovine (n = 11), porcine (n = 1) and rodent tails (n = 3) were collected fresh, chilled and processed within two hours. Tails were skinned and immersed in 70% (v/v) ethanol (EtOH) before removing fat and muscle. IVDs were dissected away from adjacent vertebrae leaving the endplates behind. IVDs subjected to histology and SISH were fixed for 24 h in >10× the volume of 4% (w/v) paraformaldehyde (PFA), rinsed for 3 × 5 min with cold 1× phosphate buffered saline (PBS) and dehydrated for 10 min each in 30% (v/v) EtOH/nuclease free (nf) 1× PBS followed by 50% (v/v) EtOH/nf 1× PBS, 70% (v/v) EtOH/nf water, 90% (v/v) EtOH/nf water, 2 × 100% (v/v) EtOH and 3× undiluted Histoclear. Tissue samples were embedded in paraffin and cut at 7um on a rotary microtome (Zhao et al., 2003). Bovine and porcine tails were retrieved from local abattoirs. All procedures were in accordance with ethical standards of Clarkson University. The study did not contain live human participants.

2.2. Non-enzymatic isolation of bovine AF, TZ and NP cells

After tail immersion in 10% (w/v) povidone-iodine, fresh IVDs were isolated as described above, dipped in 70% (v/v) EtOH and each rinsed in three sequential baths of fresh 1xPBS/10% (v/v) Gentamicin. AF, TZ and NP tissue were separated in a laminar airflow hood under sterile conditions using sterile fine scissors and razor blades. The separated AF, TZ and NP pieces were placed on uncoated 35 mm plastic culture dishes (Falcon) or 0.1% (w/v) gelatin coated 35 mm culture dishes (Falcon), provided with 1 ml sterile filtered undiluted FBS-HI, 10% (v/v) Gentamicin and 5ug/ml Amphotericin B (all GIBCO) and incubated at 37 °C, 5% (v/v) CO_2 and normal oxygen conditions as demonstrated for NP tissue pieces on 0.1% gelatin coating (Fig. 2E) and similar to AF cell isolation (Kraus and Lufkin, 2016). All dished were grouped in secondary containers by tissue type and handled separately. After 24 h of incubation 1 ml of standard D10 growth medium (1× DMEM with 4.5 g/l glucose, 1× Glutamax, 1× Pyruvate, 1× non essential amino acids, 10% (v/v) HI-FBS, 0.48% (v/v) Gentamicin (all GIBCO), and 0.12 mM beta-mercapthoethanol (Sigma)) with additional 5ug/ml Amphotericin B was added to each culture dish. After additional 48 h the tissue pieces were removed, fixed in 4% (w/v) PFA, sectioned and subjected to histology. Cells were expanded in fresh D10 medium. AF, TZ and NP derived cell lines were maintained separately and could be passaged for more than 10 times using 0.05% Trypsin/EDTA (GIBCO). Early and late passages were plated at 10^2 or 10^3 cells per well of a 96-well plate depending on the assay. AF, TZ and NP derived cell lines were subjected to differentiation medium to test for chondrogenic (50 ug/ml ascorbic acid, 40ug/ml proline, adapted from (Andriamanalijaona et al., 2008)) and lipogenic (1× ITS or 1× ITS + 3 (Sigma) adapted from (van den Akker et al., 2014)) potential in the absence or presence of dexamethasone (dex, Sigma) (chondrogenic 0.1 uM and lipogenic 1 uM adapted from (Liu et al., 2011)) and subjected to PISH and histology. Growth factors were provided via Gibco HI-FBS.

2.3. Pellet culture of AF, TZ and NP derived cells

Roughly, 10^5 cells were pelleted in 15 ml conical tubes for 5 min at 220 x g and cultured for 21 days under chondrogenic media, which was changed every three days. Individual nodules formed that were aspirated and transferred to a 1.5 ml Eppendorf tube, washed with 1× PBS and fixed over night in 1.5 ml 4% (w/v) PFA. Nodules were rinsed for 3 × 5 min with 1× PBS and dehydrated for 10 min each in 30% (v/v) EtOH/1× PBS followed by 50% (v/v) EtOH/1xPBS, 70% (v/v) EtOH/water, 90% (v/v) EtOH/water, 2 × 100% (v/v) EtOH and 3 x undiluted Histoclear. Individual nodules were then paraffin embedded and sectioned at 7 um on a rotary microtome. Sections (AF: n = 7; TZ: n = 7; NP: n = 4) were subjected to Mallory's Tetrachrome stain as described below.

2.4. RNA *in situ* hybridization

Section RNA *in situ* hybridization (SISH) (Kraus and Lufkin, 1999) on adult bovine IVDs was adapted from (Kraus et al., 2013, 2014b) using 62 °C as hybridization temperature. SISH has been carried out as multiple independent experiments (n) with two or more 7um paraffin IVD cross sections per slide per experiment: (*Col1a1*: n = 3; *Col2a1* n = 23; *Sox9* n = 5; *Acan* n = 3; *Bmp4* n = 4; *Oct4* n = 4; *MyoD* n = 3; *Krt19* n = 4). Plate *in situ* hybridization (PISH) was carried out as described (Kraus et al., 2015) hybridizing at 62 °C. Essentially, bovine cells derived from AF, TZ or NP tissue were cultured in flat bottom 96 well plates and fixed with 200ul 4% (w/v) PFA for at least 20 min, followed by 3 × 5 min rinses with nf 1× PBS

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