

CELLS UNDER PRESSURE – THE RELATIONSHIP BETWEEN HYDROSTATIC PRESSURE AND MESENCHYMAL STEM CELL CHONDROGENESIS

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Abstract

Early osteoarthritis (OA), characterised by cartilage defects, is a degenerative disease that greatly affects the adult population. Cell-based tissue engineering methods are being explored as a solution for the treatment of these chondral defects. Chondrocytes are already in clinical use but other cell types with chondrogenic properties, such as mesenchymal stem cells (MSCs), are being researched. However, present methods for differentiating these cells into stable articular-cartilage chondrocytes that contribute to joint regeneration are not effective, despite extensive investigation. Environmental stimuli, such as mechanical forces, influence chondrogenic response and are beneficial with respect to matrix formation. *In vivo*, cartilage is subjected to multiaxial loading involving compressive, tensile, shear and fluid flow. The cellular response and tissue formation upon loading are being intensively studied in the cartilage tissue-engineering research field. The study of the effects of hydrostatic pressure on cartilage formation belongs to the large area of mechanobiology. During cartilage loading, interstitial fluid is pressurised and the surrounding matrix delays pressure loss by reducing fluid flow rate from pressurised regions. This fluid pressurisation is known as hydrostatic pressure, where a uniform stress around the cell occurs without cellular deformation. *In vitro* studies, examining chondrocytes under hydrostatic pressure, have described its anabolic effect and similar studies have evaluated the effect of hydrostatic pressure on MSC chondrogenesis. The present review summarises the results of these studies and discusses the mechanisms through which hydrostatic pressure exerts its effects.

Keywords: Cartilage, mesenchymal stem cells, chondrogenesis, hydrostatic pressure, mechanobiology, early osteoarthritis.

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Introduction

Articular cartilage lines the surface of synovial joints, providing a smooth and lubricated joint surface that facilitates load-bearing. The specialised cells that create and are located within this tissue are known as chondrocytes. The primary matrix components produced by these cells includes aggrecan (a large proteoglycan) and collagen II. Chondrocyte morphology and matrix distribution within the

tissue creates an anisotropic structure (Fig. 1a,b). Anisotropy is seen not just from the cartilage surface to the subchondral bone (in the form of superficial, middle/transitional, deep and calcified zones) but also from the cell outward (pericellular, territorial and interterritorial regions) (Buckwalter *et al.*, 2005; Sophia-Fox *et al.*, 2009).

The superficial zone of the tissue contains a large proportion of collagen molecules interspersed with a low concentration of proteoglycans relative to the

rest of the tissue. Chondrocytes are flattened and aligned parallel to the cartilage surface. Cells are larger and more spherical in the middle and deep zones of the cartilage. The thickness of collagen II fibrils increases with depth and orientation is altered to be perpendicular relative to those found in the upper layers. There is also an increase in aggrecan concentration with depth of the tissue (Fig. 1a; reviewed by Buckwalter *et al.*, 2005; Sophia Fox *et al.*, 2009). The high net negative charge of the glycosaminoglycan chains on these large proteoglycans attracts water and positive ions (*e.g.* potassium and sodium). However, the water concentration decreases towards the deep zone due to matrix restrictions, leading to an increased swelling pressure, enabling cartilage to withstand high compressive loads during joint loading (Maroudas and Bannan, 1981; Mow *et al.*, 1984; Urban, 1994).

The anisotropy outwards from the chondrocyte is defined by a pericellular matrix, or chondron, which contains high concentrations of proteoglycans (*e.g.* perlecan and biglycan), other non-collagenous proteins and non-fibrillar collagens including collagen VI and IX. Collagen VI defines the boundary of the chondron. Surrounding this area is the territorial matrix, which is composed of larger collagen fibrils connected to both the pericellular matrix and the interterritorial matrix. The collagen in the territorial matrix is thought to protect the chondrocytes during mechanical loading. The interterritorial area incorporates the described cartilage zones and is the main contributor to cartilage biomechanics (Fig. 1b; Buckwalter *et al.*, 2005; Mow *et al.*, 1984; Sophia Fox *et al.*, 2009).

Articular cartilage can undergo progressive degeneration due to a variety of aetiologies, resulting in osteoarthritis (OA). One of the early events in OA involves collagen fibrillation in the superficial layer that leads to loss of proteoglycans and increase in water content. Inflammatory cytokines stimulate the expression of matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS) that degrade the tissue (Goldring, 2000; Goldring *et al.*, 2011). Chondrocytes begin to proliferate, form clusters and induce matrix formation in response to tissue damage. Despite this initial repair phase, chondrocytes reduce their proliferative and anabolic response as the degeneration continues, leading to pain and changes in joint function, which are clinical indicators of OA (Buckwalter *et al.*, 2005; Madry *et al.*, 2012).

An increased risk of OA within the knee joint is associated with previous joint injury, excessive repetitive loading, joint dysplasia and meniscus removal. These described conditions result in abnormal cartilage loading and, thus, lead to degeneration. According to the Deutsche Gesellschaft für Orthopädie und Unfallchirurgie (DGOU) registry, between October 2013 and June 2014, 60 % of treated cartilage defects were degenerative, whilst a recent multi-centre study showed that from 400 patients, approximately 40 % had chondral

injuries resulting from degenerative conditions (Angele *et al.*, 2015; Niemeyer *et al.*, 2015). In classifying the forms of OA that can be treated, recent studies have used the term 'early OA' (Luyten *et al.*, 2012; Madry *et al.*, 2016; Madry *et al.*, 2012). Focal early OA is focussed on the defect and the immediate surrounding environment. In clinical terms, the focal lesion [International Cartilage Regeneration & Joint Preservation Society (ICRS) grade 3-4] is degenerative, whilst there is minimal degeneration (ICRS grade 1-2) in the surrounding cartilage area (defined by Madry *et al.*, 2016). This condition may be treated using regenerative medicine or tissue engineering therapies. However, a high failure and re-operation rate is observed for focal degenerative defects as compared with those resulting from traumatic or chronic injury upon treatment using autologous chondrocyte implantation (ACI). A potential reason for their poor outcome is the surrounding inflammatory environment that impairs cell-based solutions. In particular, IL-1 β has been shown to be a negative predictor for ACI treatment post-surgery (Angele *et al.*, 2015; McNulty *et al.*, 2013). Thus, a primary goal for clinicians and scientists is to develop regenerative options that can be used to treat both focal and diffuse early OA in this challenging environment.

Tissue engineering strategies may be useful for treating early osteoarthritic defects. The primary cell-based tissue engineering strategy for treating the described aetiology involves the use of autologous articular-cartilage chondrocytes (Brittberg *et al.*, 1994). Alternative cell types such as mesenchymal stem cells (MSCs) from a variety of sources are being explored. These cells can be isolated from bone marrow, adipose tissue (liposuction or intrapatellar fat pad) or synovium with minimal donor site morbidity (Caplan, 1991; Gimble and Guilak, 2003; Pittenger *et al.*, 1999; Sakaguchi *et al.*, 2005). The use of pre-formed chondrogenic implants requires *in vitro* MSC chondrogenesis to be stimulated. Initially, this was performed through the creation of pellets or micromasses and addition of the stimulatory factor transforming growth factor beta 1 (TGF- β 1) (Johnstone *et al.*, 1998; Yoo *et al.*, 1998), which upregulates the transcription factors SOX9, SOX5 and SOX6 (Lefebvre *et al.*, 2001; Lefebvre *et al.*, 1997). The expression of these transcription factors, and specifically SOX9, leads to collagen II and aggrecan expression and synthesis (Fig. 1c). Since the development of this method for the chondrogenic differentiation of stem/progenitor cells, the field has expanded to include cellular chondrogenesis within scaffolds/biomaterials to create tissue constructs of a clinically relevant size for filling cartilage defects within patient's joints. Unfortunately, these cell-biomaterial constructs usually have inferior biochemical and mechanical properties when compared with the native tissue (Erickson *et al.*, 2009). Moreover, in both pellets and scaffolds, markers of chondrocyte hypertrophy (collagen type X, MMP-

13) have been detected and, upon implantation *in vivo*, ectopic bone formation can occur (Pelttari *et al.*, 2006). Strategies to prevent hypertrophy and produce a stable chondrocyte phenotype with its defined extracellular matrix are the goal of *in vitro* cartilage tissue engineering.

Scientists have attempted to attain stable cartilage formation with environmental stimuli relevant to the *in vivo* situation, *e.g.* biomechanical stimulation, lower oxygen tension and/or addition of growth factors. Chondrocytes and MSCs have been shown to respond to mechanical loading and alter their matrix production in response to different stimuli (reviewed by Anderson and Johnstone, 2017; Huang *et al.*, 2010a; O'Connor *et al.*, 2013). One of the simplest stimuli that is involved in cartilage biomechanics is hydrostatic pressure. This involves fluid pressurisation and has been shown to increase tissue formation upon application to articular cartilage chondrocytes (reviewed by Elder and Athanasiou, 2009; Salinas *et al.*, 2018). The present review summarises the literature and evaluates the effects of hydrostatic pressure on MSC chondrogenesis, summarising the outcomes of the various studies and the pathways that have been identified to be part of the cellular response to it.

Generation of hydrostatic pressure in articular cartilage

Hydrostatic pressure is the application of a uniform stress without cellular deformation. *In vivo*, this is generated by pressurisation of interstitial water within articular cartilage. Theoretical models (*e.g.* biphasic theory) predicting articular cartilage mechanics show that upon loading, cartilage develops a resistance to fluid flow and generates a pressure within the matrix: the hydrostatic pressure. These models predict that approximately 90 % of load is initially borne by fluid pressurisation between cartilage layers, which contributes to its viscoelastic properties (Bachrach *et al.*, 1998; Carter and Wong, 2003; Soltz and Ateshian, 1998; Soltz and Ateshian, 2000). Soltz and Ateshian (1998, 2000) demonstrated the generation of pressure within articular cartilage in an experimental setting. They found that the interstitial fluid or water within the matrix is subjected to hydrostatic pressure, whilst the collagen-proteoglycan matrix helps delay pressure loss through retarding the rate of fluid flow away from pressurised regions. It may be postulated that the highest hydrostatic pressure occurs below the superficial layer due to the presence of proteoglycans attracting water within these layers during compression (Fig. 1a). This form of loading causes no cellular or tissue deformation and has been hypothesised to play a role in protecting chondrocytes and articular cartilage matrix from excessive loads generated within the joint (Soltz and Ateshian, 1998). Measurements of hydrostatic pressure during loading suggest that

the peak pressure can be up to 12 MPa (Soltz and Ateshian, 2000). In contrast, compression and shear loading, also contributory to cartilage biomechanics, involve both cellular and tissue deformation. Thus, hydrostatic pressure applies its mechanical load in a distinct manner.

Pressure directs chondrogenic fate during joint development (Carter and Wong, 2003; Giorgi *et al.*, 2014). Giorgi *et al.* (2014) found in a computational model that dynamic/cyclic hydrostatic pressure promotes cartilage formation, whereas static pressure induces bone formation during prenatal joint morphogenesis. Theoretical models have also suggested that pressure helps to maintain the chondrocyte phenotype within the cartilage (Carter and Wong, 2003). Thus, according to these models, hydrostatic pressure drives chondrogenesis during the embryonic and mesenchymal phase, whilst helping to maintain chondrocyte phenotype at maturity. A previous review addressed the effects of hydrostatic pressure on chondrocytes (Elder and Athanasiou, 2009). In summary, chondrocytes subjected to hydrostatic pressure demonstrate an enhanced chondrocyte matrix production, particularly between 0.1 and 10 MPa under either static or cyclic settings, as compared with unloaded controls. Recent investigations have also begun to examine the effects of hydrostatic pressure on MSCs from a variety of animal and tissue sources (Table 1-3).

Hydrostatic pressure and MSC chondrogenesis

The first study to investigate the effect of hydrostatic pressure on MSCs was performed by Angele *et al.* (2003) (Table 1a,b). Pressure was applied to MSC pellets during the first week of culture using a loading regime within a physiological range (0.55-5 MPa at 1 Hz; 4 h/d). The results demonstrated that there is an increase in glycosaminoglycans (GAG) and collagen matrix synthesis after loading at day 14 and 28, whilst pellets loaded only on day 1 and 3 do not increase matrix deposition with respect to the unloaded control (Angele *et al.*, 2003). Following this study, similar studies used continuous hydrostatic pressure (0.5-5 MPa, 0.5 Hz) applied to adipose-derived MSCs seeded within a collagen type I scaffold with medium replenishment during the loading phase. These latter studies showed that SOX9, collagen type II alpha 1 chain (COL2A1) and aggrecan (ACAN) are upregulated at weeks 2 and 4, whilst the early hypertrophy marker, COL10A1, is upregulated in the loaded groups from weeks 3 onwards (Ogawa *et al.*, 2009). Other investigations obtained similar results, whereby chondrogenic (SOX9, COL1A1, COL2A1 and ACAN) and matrix synthesis (GAG and COL) gene are increased upon application of hydrostatic pressure following either 14 or 21 d of application (Carroll *et al.*, 2014; Correia *et al.*, 2012; Elder *et al.*, 2008; Miyanishi *et al.*, 2006; Ogawa *et al.*, 2009; Ogawa *et al.*, 2015; Saha *et al.*, 2017; Sakao *et al.*, 2008; Steward *et al.*, 2014; Steward

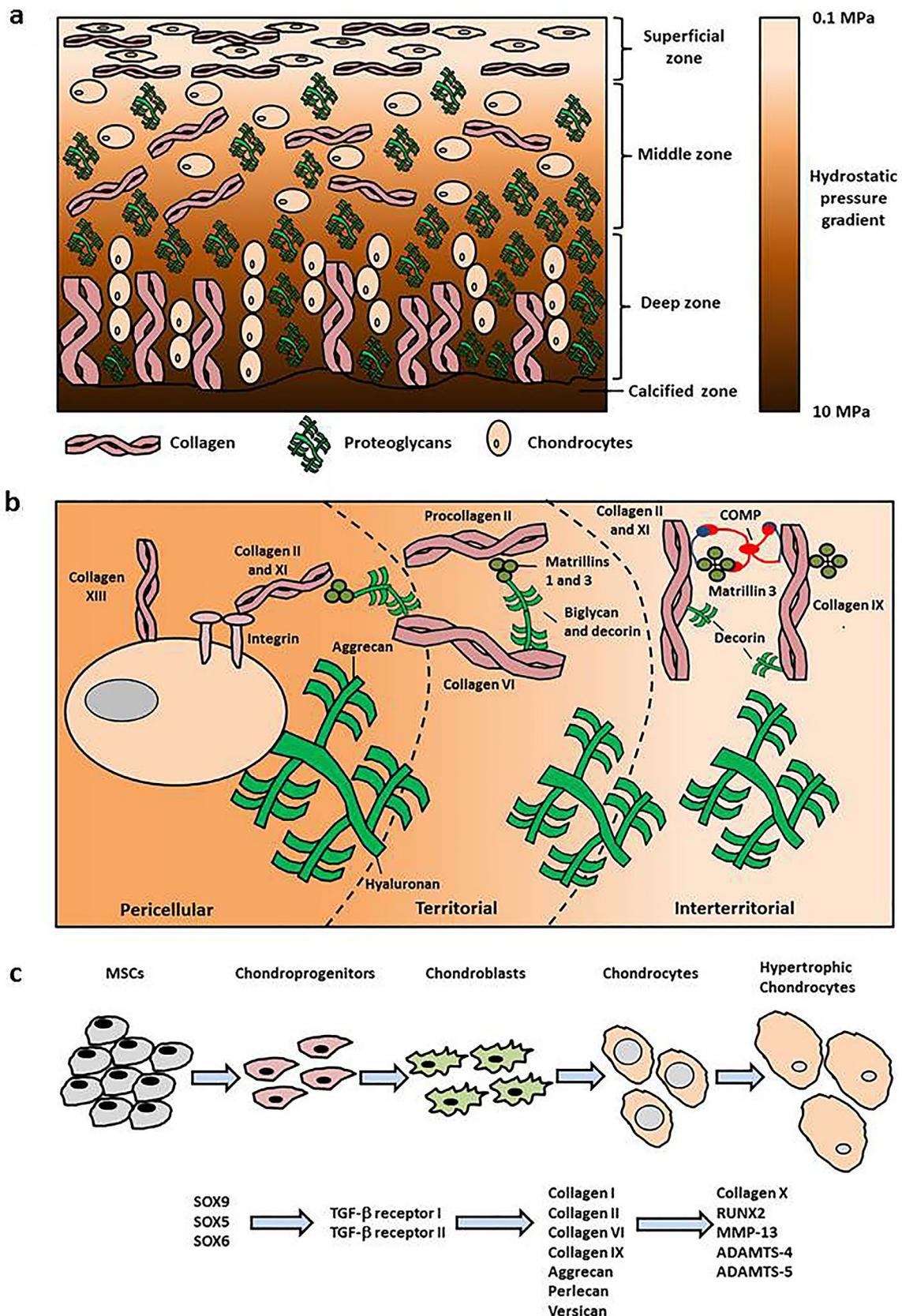


Fig. 1. Schematic diagram. (a) Zones of articular cartilage depicting the arrangement and orientation of chondrocytes, collagens and proteoglycans within the extracellular matrix and the changes in hydrostatic pressure. (b) A simplified diagram of the matrix regions surrounding the chondrocyte, specifically the pericellular, territorial and interterritorial matrix and the specific matrix molecule arrangements within these regions. (c) The processes involved in MSC chondrogenesis with respect to chondrocyte maturation and the genes involved at each stage of the process.

et al., 2016; Steward *et al.*, 2012; Steward *et al.*, 2013; Wagner *et al.*, 2008; Ye *et al.*, 2014; Zhao *et al.*, 2015) (Table 1-3). Loading magnitude and duration, length of chondrogenic pre-culture or preconditioning prior to pressure application and inclusion/exclusion of TGF- β influence the chondrogenic response of MSCs.

Loading magnitude and duration of pressure on MSC chondrogenesis

Loading magnitudes ranging from 0.1-10 MPa enhance MSC chondrogenesis (Angele *et al.*, 2003; Correia *et al.*, 2012; Karkhaneh *et al.*, 2014; Miyanishi *et al.*, 2006; Ogawa *et al.*, 2009; Vinardell *et al.*, 2012). Miyanishi *et al.* (2006b) showed that there is a load-dependent matrix gene expression and deposition, whereby 0.1-1 MPa loading can increase *SOX9*, *COL2A1* and *ACAN* expression, although peak expression for the latter two genes occurs at 10 MPa. The latter magnitude induces a significant increase in GAG and collagen matrix formation, indicating that different magnitudes elicit differential responses in MSCs at the gene and protein level (Table 1a,b).

Correia *et al.* (2012), under similar conditions and using adipose MSCs (4 h/d at 0.5 Hz), reproduced these results: high peak magnitudes (7.5 MPa) increase matrix deposition from adipose MSCs, whilst low magnitudes (0.4 MPa) upregulate *COL2A1*. However, other investigations have also shown that 0.5-1 MPa hydrostatic pressure is sufficient to enhance MSC chondrogenesis, although no comparisons are made with other peak pressures (Ogawa *et al.*, 2009; Saha *et al.*, 2017; Wagner *et al.*, 2008). In summary, although loading magnitude results in differing gene expression profiles and matrix production response, pressure generally induces an anabolic effect on MSC chondrogenesis.

There has not been extensive research on the effects of pressure duration on chondrogenic MSCs. The loading duration in these and most of the studies investigating hydrostatic pressure on MSC chondrogenesis is 4 h/d. Based on the current literature, it can be stated that pressure can enhance chondrogenesis when applied between 1-4 h/d but there is no information on the optimal loading period (Elder *et al.*, 2008; Karkhaneh *et al.*, 2014; Luo and Seedhom, 2007; Meyer *et al.*, 2011; Sakao *et al.*, 2008; Ye *et al.*, 2014; Zhao *et al.*, 2016; Zhao *et al.*, 2015).

TGF- β presence and endogenous production in pressure-stimulated MSC chondrogenesis

Most studies of pressure-induced chondrogenesis used MSCs cultured in the presence of 10 ng/mL TGF- β . Enhanced chondrogenesis as compared with unloaded controls is documented with 1 ng/mL TGF- β using either bone-marrow-, synovium- or adipose-derived MSCs (Miyanishi *et al.*, 2006; Vinardell *et al.*, 2012). However, larger increases in gene expression and matrix formation are observed at 10 ng/mL TGF- β with hydrostatic pressure applied.

Miyanishi *et al.* (2006a) examined the application of hydrostatic pressure without TGF- β on bone marrow MSCs. At 10 MPa, 1 Hz for 4 h/d, they detected significant increases in *SOX9*, *COL2A1* and *ACAN* expression and matrix deposition (aggrecan and collagen II staining) following 14 d of loading. Finger *et al.* (2007) examined this phenomenon and found that *SOX9* is upregulated in MSCs upon loading at 7.5 MPa hydrostatic pressure. However, a follow-up study showed that despite an upregulation in both *SOX9* and *ACAN* expression during the first week, there is no chondrogenic gene expression at later time points due to loss of cell viability (Puetzer *et al.*, 2013). In agreement with these results, Safeshaken *et al.* (2012), using a pellet culture model, demonstrated no increase in chondroinduction without TGF- β addition using a loading regime (0-5 MPa at 0.5 Hz for 4 h/d) for 10 d only. Thus, whether hydrostatic pressure alone is sufficient to induce chondrogenesis to any significant degree remains unresolved (Carroll *et al.*, 2014; Finger *et al.*, 2007; Li *et al.*, 2012; Li *et al.*, 2009; Puetzer *et al.*, 2013; Vinardell *et al.*, 2012; Ye *et al.*, 2014) (Table 2).

Specially designed multi-axial mechanical loading systems that simulate knee biomechanics and involve compression, shear and hydrostatic pressure stimulate endogenous TGF- β (Gardner *et al.*, 2016b; Li *et al.*, 2010; Safshekan *et al.*, 2012; Schatti *et al.*, 2011). Importantly, the application of mechanical forces, and more specifically shear loading, induces conversion of TGF- β from its latent to active form (Ahamed *et al.*, 2008; Albro *et al.*, 2012; Gardner *et al.*, 2016b). Hydrostatic pressure alone stimulates active TGF- β secretion from chondrogenic MSC without TGF- β supplementation (Li *et al.*, 2012; Maxson and Burg, 2012; Ye *et al.*, 2014). However, it should be noted that cells subjected to hydrostatic pressure are either stimulated with TGF- β prior to loading (Li *et al.*, 2012; Ye *et al.*, 2014) or cultured in conditioned medium containing TGF- β under pressure (Maxson and Burg, 2012). This contrasts with the described multi-axial loading studies, in which MSCs undergo chondrogenesis in the absence of TGF- β during loading (Gardner *et al.*, 2016b; Li *et al.*, 2010; Schatti *et al.*, 2011). Contrary to previous hydrostatic pressure publications, Vinardell *et al.* (2012) demonstrated that without TGF- β pre-stimulation from the outset, pressure alone do not induce endogenous TGF- β production from MSCs. Thus, based on these studies, TGF- β pre-differentiation may be required for pressure alone to induce endogenous production, although the mechanism needs to be fully understood.

Chondrogenic MSC pre-differentiation and response under hydrostatic pressure

Loading of chondrogenic pre-cultured MSCs at different stages of differentiation has been examined in mechanical loading systems involving

Table 1a. Summary of the effects of hydrostatic pressure on MSC chondrogenesis under different loading regimes.

Publication	Cell source	Pellet/scaffold	Loading regime	Principal findings
Angele <i>et al.</i> , 2003	Human bone marrow MSCs	Pellet	Cyclic, 0.555 MPa, 1 Hz, 4 h/d, loading on day 1, 3 or 17	Pellet size, GAG and collagen synthesis significantly increase from day 17 after loading application as compared to unloaded control. No effect of loading on single loading days.
Miyaniishi <i>et al.</i> , 2006b	Human bone marrow MSCs	Pellet	Cyclic, 0.1, 1, 10 MPa at 1 Hz, 4 h/d, 14 d continuously	Increased <i>SOX9</i> and <i>ACAN</i> expression peaking at 10 MPa. GAG synthesis upregulated at 1 and 10 MPa, whilst collagen synthesis increases only at 10 MPa.
Elder <i>et al.</i> , 2008	Murine embryonic cell line (CH310T1/2)	Pellet	Cyclic, 0.55 MPa, 1 Hz, 3 h/d, 3 d	Enhanced <i>SOX9</i> and <i>ACAN</i> expression upon loading.
Sakao <i>et al.</i> , 2008	Rabbit synovium MSCs	Scaffold (4 × 10 ⁶ cells/mL in 1.2 % alginate beads – synovium MSCs)	Cyclic, 15 MPa, 0.5 Hz, 1 h/d	<i>SOX9</i> and <i>COL2</i> expression and GAG synthesis upregulated upon loading.
Wagner <i>et al.</i> , 2008	Human bone marrow MSCs	Scaffold (1 × 10 ⁶ cells/mL; collagen type I construct)	Cyclic, 1 MPa, 1 Hz, 4 h/d for 10 d	Upregulated <i>SOX9</i> , <i>ACAN</i> , <i>COL1A1</i> and <i>COL2A1</i> expression. More GAG matrix staining in loaded constructs.
Ogawa <i>et al.</i> , 2009	Human adipose MSCs	Scaffold (1 × 10 ⁶ cells/mL; collagen type I construct)	Cyclic, 0.5 MPa, 0.5 Hz, week 1 loaded (continuously/no rest under load)	<i>SOX9</i> , <i>ACAN</i> , <i>COL2A1</i> and <i>COLX</i> expression increase under loading More collagen II and keratin sulphate staining after loading.
Correia <i>et al.</i> , 2012	Human adipose MSCs	Scaffold (10 × 10 ⁶ cells/mL; collagen type I construct)	Cyclic; high: 5 MPa, 0.5 Hz, 4 h/d, 5 d/week; low: 0.4 MPa, 0.5 Hz, 4 h/d, 5 d/week	No difference in <i>SOX9</i> expression under either loading regime; <i>ACAN</i> expression peaks at 5 MPa, whilst <i>COL2A1</i> at 0.4 MPa. More GAG accumulation and matrix staining under high hydrostatic loading.
Steward <i>et al.</i> , 2012	Porcine bone marrow MSCs	Scaffold (15 × 10 ⁶ cells/mL; 2 % agarose or fibrin)	Cyclic, 10 MPa, 1 Hz, 4 h/d, 5 d/week for 5 weeks 1 ng/mL or 10 ng/mL TGF-β	GAG synthesis increases and collagen I decreases in fibrin hydrogels upon loading, whilst more pericellular matrix is observed in agarose gels. ALP activity suppressed upon loading in the presence of 10 ng/mL TGF-β in agarose gels.
Steward <i>et al.</i> , 2013	Porcine bone marrow MSCs	Scaffold [15 × 10 ⁶ cells in 1 %, 2 % or 4 % (w/v) agarose]	Cyclic, 10 MPa, 1 Hz, 4 h/d, 5 d/week	<i>SOX9</i> , <i>COL2A1</i> and <i>ACAN</i> expression increases upon loading within stiffer hydrogels. Application of integrin inhibitor (RGDS) reduces chondrogenic response under loading.
Carroll <i>et al.</i> , 2014	Human bone marrow MSCs and infrapatellar fat pad MSCs	Scaffold (20 × 10 ⁶ cells/mL; 2 % agarose)	Cyclic, 10 MPa, 1 Hz, 4 h/d, 5 d/week for 5 weeks	Pressure increases GAG and collagen synthesis in both cell types. More collagen II staining in bone marrow MSCs. Reduced alizarin red staining under pressure; potentially preventing hypertrophy dynamic and equilibrium modulus increases upon loading application.

compression and/or in combination with shear (reviewed by O'Connor *et al.*, 2013). These studies show that the longer the pre-differentiation period prior to loading the larger the increase in cartilage gene expression and matrix production upon stimulation. Continuous loading at the start of culture appears to be detrimental, indicating that a period of pre-culturing or pre-differentiation prior to loading is beneficial for MSC chondrogenesis

(Huang *et al.*, 2010b; Thorpe *et al.*, 2010). This indicates the importance of early matrix formation, especially pericellular matrix, to enable transmission of the compressive force (mechanotransduction), mimicking that produced in *in vivo* cartilage.

Similar results have been found varying the point of hydrostatic loading on differentiating chondrogenic MSCs (Li *et al.*, 2012; Li *et al.*, 2016; Luo and Seedhom, 2007; Ogawa *et al.*, 2015; Safshekan

Table 1b. Summary of the effects of hydrostatic pressure on MSC chondrogenesis under different loading regimes.

Publication	Cell source	Pellet/scaffold	Loading regime	Principal findings
Steward <i>et al.</i> , 2014	Porcine bone marrow MSCs	Scaffold (15 × 10 ⁶ cells/mL; 4 % agarose)	Cyclic, 10 MPa, 1 Hz, 4 h/d, 5 d/week	Pressure enhances GAG and collagen II matrix deposition. Calcium signalling inhibition prevents anabolic response but stretch-activated channels inhibition does not affect response.
Ye <i>et al.</i> , 2014	Rat bone marrow MSCs	Pellets 17β-oestradiol (E ₂); tamoxifen –ERα blocker	Cyclic, 90 kPa for 1 h/d	SOX9, ACAN and COL2A1 expression upregulated and GAG synthesis increased under loading. 17β-oestradiol and loading enhance response <i>via</i> JNK phosphorylation and expression, whilst presence of tamoxifen prevents load-induced chondrogenic response.
Ogawa <i>et al.</i> , 2015	Human adipose MSCs	Scaffold (1 × 10 ⁶ cells/mL; collagen type I construct)	Cyclic, 0.5 MPa, 0.5 Hz, week 1 loaded, week 1 and 3 loaded, continuous loading	Week 1 loading is sufficient to induce chondrogenesis. SOX9, ACAN, COL2A1, COLXA1 and MMP3 and MMP13 upregulated upon loading – more GAG and collagen matrix synthesis.
Zhao <i>et al.</i> , 2015	Rat bone marrow MSCs	Pellet	90 kPa cyclic, 1 h, 4 weeks continuous loading	SOX9, ACAN and COL2A1 expression and GAG synthesis upregulated with Rac1 agonist and RhoA antagonist under loading.
Steward <i>et al.</i> , 2016	Porcine bone marrow MSCs	Scaffold [15 × 10 ⁶ cells in 1 % or 4 % (w/v) agarose]	Cyclic, 10 MPa, 1 Hz, 4 h/d, 5 d/week	Pressure increases extracellular ATP production through hemichannels and facilitates anabolism under loading. Inhibition of hemichannels, P-receptors or extracellular ATP, suppressed chondrogenic response – vimentin structure unaltered in the presence of inhibitors.
Saha <i>et al.</i> , 2017	Murine embryonic limb bud cells	Scaffold (2 × 10 ⁷ cells/mL; 1.8 % agarose)	Cyclic, 1 MPa, 1 Hz, 4 h/d, 8 d loading	SOX9 expression increases under loading. COLXA1 and IHH expression decreases under loading – collagen X staining also reduced.

et al., 2012; Ye *et al.*, 2014). In general, application of pressure after a period of pre-differentiation (7 or 21 d) enhances chondrogenesis as compared with unloaded controls (Table 3a,b). Meyer *et al.* (2011), using a cell-agarose model, studied whether hydrostatic pressure, used either at the onset or delayed (21 d), resulted in enhanced chondrogenesis. In contrast to compressive and shear loading studies, application of hydrostatic pressure at the onset produces more matrix formation (GAG, collagens I, II) and increased mechanical properties (Young's and dynamic modulus) as compared to pre-differentiated MSCs. Despite no in-depth studies regarding the effect of different periods of pre-differentiation, it may be hypothesised that hydrostatic pressure has a larger effect on MSC chondrogenesis during its early stages as suggested in computational/theoretical models, whilst compressive loading exerts its influence on pre-differentiated MSCs, specifically at later stages of chondrogenesis.

Hypertrophic MSC chondrogenesis and hydrostatic pressure

Some investigations examining pressure-stimulated chondrogenesis found that the hypertrophic markers *COL10A1*, *MMP-13* and Indian hedgehog (*IHH*) are

upregulated (Li *et al.*, 2012; Ogawa *et al.*, 2009; Ogawa *et al.*, 2015). However, other investigations have detected downregulation in gene expression of these markers (Liu *et al.*, 2014; Saha *et al.*, 2017; Steward *et al.*, 2012; Vinardell *et al.*, 2012). Furthermore, when Carroll *et al.* (2013) stimulated encapsulated MSCs in normal chondrogenic medium for 3 weeks and, then, in hypertrophic medium for 2 weeks, hydrostatic pressure helped to suppress MSC calcification during culture in hypertrophic medium, whilst increasing collagen and glycosaminoglycan content. Saha *et al.* (2017), using murine embryonic limb bud cells subjected to hydrostatic loading, had similar results and showed reduced collagen X protein deposition. Given the conflicting results, the effects of hydrostatic pressure on chondrocyte hypertrophy need further study for conclusive statements to be made about its effects.

Signalling pathways under the influence of hydrostatic pressure

Hydrostatic pressure demonstrates a pro-chondrogenic effect, whereby matrix synthesis is enhanced upon stimulation. The pathways that

Table 2. Summary of publications examining the influence of TGF- β on pressure-induced chondrogenesis.

Publication	Cell source	Pellets/scaffold	Loading regime	TGF- β conditions	Principal findings
Miyanishi <i>et al.</i> , 2006a	Human bone marrow MSCs	Pellet	Cyclic, 1 Hz, 10 MPa, 4 h/d, 14 d, continuously	0 ng/mL 10 ng/mL	<i>SOX9</i> , <i>COL2A1</i> and <i>ACAN</i> increased expression under pressure with or without TGF- β . More matrix synthesis in the presence of both TGF- β and pressure.
Finger <i>et al.</i> , 2007	Bone marrow MSCs	Scaffold (9×10^6 cells/mL; 2 % agarose)	Steady cyclic loading, 7.5 MPa, 1 Hz, 4 h/d (14 d) or ramped cyclic loading starting at 0.5 MPa, increase by 0.5 MPa/d	0 ng/mL	<i>COL1A1</i> and <i>SOX9</i> expression increase under both loading regimes. No collagen type II and aggrecan expression.
Maxson and Burg, 2012	Murine bone marrow MSCs	Scaffold (2×10^6 cells/mL; 2 % agarose)	Cyclic, 300 kPa, 0.5 Hz, 0.7 mL/min medium perfusion	Conditioned medium from chondrogenic MSCs, no TGF- β application under load	<i>SOX9</i> , <i>COL2A1</i> and <i>ACAN</i> increased expression under pressure. More GAG synthesis under mechanical load with increased TGF- β secretion.
Vinardell <i>et al.</i> , 2012	Porcine synovium membrane MSCs and infrapatellar fat pad MSCs	Pellets	Cyclic, 10 MPa, 1 Hz, 4 h/d, 2 weeks	0, 1, 10 ng/mL	<i>SOX9</i> , <i>ACAN</i> , <i>COL2A1</i> expression upregulated in the presence of TGF- β . GAG synthesis increases upon loading in the presence of 1 ng/mL TGF- β . No effect on collagen synthesis. No increase in endogenous TGF- β production or TGF- β receptor II expression.
Puetzer <i>et al.</i> , 2013	Human adipose MSCs and bone marrow MSCs	Scaffold (9×10^6 cells/mL; 2 % agarose)	Cyclic, 7.5 MPa, 1 Hz, 4 h/d, 21 d loading	0 ng/mL	<i>SOX9</i> , <i>ACAN</i> and <i>COMP</i> expression upregulated upon loading but no expression after 14 and 21 d.

control this response have been examined in a few studies (Fig. 2).

Cell surface receptors, membrane channels and downstream signalling

Mechanotransduction involves transfer of the mechanical force (*e.g.* compressive, tensile or hydrostatic pressure) into a signal through cell surface receptors or membrane channels during biomechanical stimulation. Few studies have examined how mechanotransduction of hydrostatic loading occurs. Due to the presence of TGF- β , TGF- β receptor I and II may have an influence on pressure-stimulated chondrogenesis with the canonical TGF- β /SMAD pathway being a candidate downstream pathway (reviewed by Cleary *et al.*, 2015). TGF- β stimulates phosphorylation of SMAD2 and SMAD3 and this, subsequently, leads to nucleus translocation and regulation of chondrogenic genes (*SOX9*, *COL2A1*, *ACAN*). However, inhibition of TGF- β receptor I only partially inhibits the anabolic response

caused by hydrostatic pressure, indicating that other pathways are involved (Li *et al.*, 2012). Non-canonical SMAD pathways have also been investigated due to their association with TGF- β -induced chondrogenesis; specifically, mitogen-activated protein (MAP) kinases including extracellular-regulated protein kinase-1 (ERK1), p38 and c-Jun N-terminal kinases (JNK) (Li *et al.*, 2009; Sakao *et al.*, 2008; Zhao *et al.*, 2016; Zhao *et al.*, 2015). Sakao *et al.* (2008) reported that application of hydrostatic pressure in the presence of ERK and p38 inhibitors does not inhibit expression of *SOX9*, *COL2A1* and proteoglycan core protein but addition of a JNK inhibitor prevents upregulation of these genes. Recent investigations have shown that hydrostatic pressure enhances phosphorylated JNK expression, leading to subsequent increases in *SOX9*, *COL2A1* and *ACAN* expression (Zhao *et al.*, 2015).

Integrins are transmembrane proteins composed of α and β sub-units that mediate cell-extracellular matrix interactions (Loeser, 2014). Integrin binding of cells to the extracellular matrix may facilitate

mechanotransduction of mechanical forces applied to cartilage (Docheva *et al.*, 2014; Loeser, 2014). Steward *et al.* (2014), using an RGDS peptide as an integrin-binding inhibitor, showed that the beneficial effects of hydrostatic pressure are inhibited in its presence. However, RGDS is a broad integrin inhibitor and the specific integrin sub-units involved were not evaluated. There are no reports of hydrostatic pressure studies with inhibition or knock-down of specific integrins to understand their specific contributions to mechanotransduction.

Oestrogen has been implicated in chondrogenesis and OA, as studies have described a higher prevalence of OA in female patients who are going through menopause and have reduced levels of oestrogen (Richette *et al.*, 2003). Replacement therapy may reduce cartilage loss but there have been conflicting results (Sahyoun *et al.*, 1999; Wluka *et al.*, 2001). Hydrostatic pressure upregulates oestrogen receptor (ER α) activity during MSC chondrogenesis, whilst application of 17 β -oestradiol (E₂), combined with pressure, enhances matrix synthesis (Fig. 2a) (Zhao *et al.*, 2016). However, tamoxifen (ER α inhibitor) application reduces chondrogenic gene expression and GAG content upon stimulation, although it has no effect on unloaded controls. Downstream of ER α activation, mitogen-activated protein kinase (MAPK) pathway and, specifically, JNKs are phosphorylated under pressure and contribute to the anabolic response. In the presence of tamoxifen, JNK is not phosphorylated under pressure, indicating that this is a downstream target of pressure-induced chondrogenesis (Zhao *et al.*, 2016). However, other downstream targets of ER α have yet to be elucidated.

Membrane channels that allow the movement of molecules both in and out of cells and between cells, altering the osmotic pressure during hydrostatic loading have been studied in chondrocytes (Hall, 1999). The author demonstrated that cation transporters (Na/K/2Cl and Na/K pumps) are inhibited in chondrocytes under hydrostatic pressure ranging from 0.1-10 MPa, indicating that other channels may be involved in increasing matrix synthesis under pressure for articular-cartilage chondrocytes. In the case of MSC chondrogenesis under the influence of hydrostatic pressure, studies have primarily focussed on channels involved in purinergic and calcium signalling. Stretch-activated calcium channels and voltage-gated calcium channels have been implicated in the mechanotransduction process. Stretch-activated channels are induced by different types of mechanical loading, including hydrostatic pressure (Mizuno, 2005; Mobasher *et al.*, 2002), whereas voltage-gated calcium channels are activated through membrane depolarisation and mediate calcium influx. In the latter case, calcium movement through these channels increases cellular calcium concentration directly or indirectly from calcium released from sarcoendoplasmic reticulum calcium stores (SERCs). Downstream, calcium initiates the activity of secondary messengers

(*e.g.* calmodulin, calmodulin kinase type II and calcineurin) that leads to a variety of cascades to be activated, particularly chondrogenic gene expression (Knight *et al.*, 2009; Steward *et al.*, 2014). These messengers are initiated under compressive and fluid flow mechanotransduction in MSCs and chondrocytes (Mizuno, 2005; Mobasher *et al.*, 2002).

Hydrostatic pressure applied during MSC chondrogenesis induces an anabolic response through voltage-gated ion channels, as inhibition prevents pressure-induced matrix synthesis, whereas inhibiting stretch-activated channels do not prevent the anabolic effect of pressure (Steward *et al.*, 2014). Use of a calcium chelator preventing intracellular calcium movement, also significantly reduces GAG and collagen synthesis upon pressure stimulation (Steward *et al.*, 2014). Thus, membrane depolarisation and calcium mobilisation are a mechanism of pressure-stimulated cartilage formation. However, the exact reason for how voltage-gated ion channels are involved in the hydrostatic pressure response requires further investigation.

A candidate pathway involved in this process and that utilises voltage-gated ion channels is the purinergic signalling. This involves hemichannels, which are membrane proteins consisting of connexins that form gap junctions and enable cell-cell communication (Millward-Salder *et al.*, 2004; Knight *et al.*, 2009). Upon mechanical stimulation, MSCs release ATP and this extracellular release through hemichannels enables it to bind to the purine receptors P2X or P2Y. P2X receptor is an ATP-gated ion channel and P2Y receptors are coupled to G-proteins to elicit a calcium response through inositol-triphosphate-mediated release from sarcoendoplasmic reticulum stores (SERCs) (Fig. 2b). The increased calcium concentration depolarises the membrane and amplifies the signal through activation of the voltage-gated ion channels and calcium release from intracellular stores (Steward *et al.*, 2014a). The authors demonstrated that inhibition of SERCs also inhibits the anabolic effects of hydrostatic pressure on MSC chondrogenesis.

Steward *et al.* (2016) studied the purinergic signalling pathway in more detail to understand its importance during hydrostatic pressure chondrogenesis. Pressure activates the pathway, as indicated by increased extracellular ATP production. However, inhibition of hemichannels, P-receptors or extracellular ATP blocks the anabolic response upon pressure stimulation, indicating its importance during pressure-induced signalling. Addition of exogenous ATP without pressure does not reproduce this behaviour, thus showing the importance of hydrostatic pressure for purinergic signalling and that other upstream mechanisms are stimulated to upregulate chondrogenic gene expression and matrix production. These latter studies suggest differences in the mechanotransduction of hydrostatic pressure *versus* compressive and multiaxial loading regimes.

Table 3a. Summary of the effects of hydrostatic pressure on pre-differentiated chondrogenic MSCs.

Publication	Cell source	Pellets/scaffold	Loading regime	Pre-differentiation period	Principal findings
Luo and Seedhom, 2007	Ovine bone marrow MSCs	Scaffold (5 × 10 ⁵ cells/mL, plasma-treated polyester)	Cyclic, 0.1 MPa, 0.25 Hz, 30 min/d, 10 d culture	28 d	DNA, GAG and collagen content increase significantly.
Li <i>et al.</i> , 2009	Rat bone marrow MSCs	Scaffold [4 × 10 ⁶ cells in 1.5 % (w/v) alginate beads]	Cyclic, 14-36 kPa, 0.25 Hz, 1 h/d, 7 d loading	7 d	<i>SOX9</i> , <i>COL2A1</i> and <i>ACAN</i> expression stimulated by loading only – elevated in the presence of TGF-β. p38 MAP kinase stimulated by loading, elevated and enhanced in the presence of TGF-β – only partially suppressed loading response in the presence of inhibitor.
Meyer <i>et al.</i> , 2011	Porcine bone marrow MSCs	Scaffold (15 × 10 ⁶ cells/mL; 2 % agarose)	Cyclic, 10 MPa, 1 Hz, 4 h/d, 5 d/week for 5 weeks Continuous (0-42 d) Delayed (22-42 d and no TGF-β)	21 d	Continuous loading increases dynamic modulus, delayed loading has no effect – increased matrix synthesis in core under loading.
Li <i>et al.</i> , 2012	Rat bone marrow MSCs	Scaffold [5 × 10 ⁶ cells in 1.5 % (w/v) alginate beads]	Cyclic, 14-36 kPa, 0.25 Hz, 1 h/d, 7 d loading	7 d	<i>SOX9</i> , <i>ACAN</i> , <i>COL2A1</i> , <i>RUNX2</i> and <i>IHH</i> expression upregulated upon loading with or without TGF-β. GAG and collagen II synthesis increased upon loading, independently of TGF-β presence – synergistic effect in its presence. Endogenous TGF-β production stimulated under loading. Canonical TGF-β/SMAD pathway not solely involved in pressure-induced chondrogenic response.
Liu <i>et al.</i> , 2012	Human infrapatellar fat pad MSCs	Pellet culture; scaffold [20 × 10 ⁶ cells in 2 % (w/v) agarose] 5 % oxygen physioxic preconditioning	Cyclic, 10 MPa, 1 Hz, 2 h/d, 5 d/week	14 d	No enhancement in matrix synthesis but mechanical stiffness increases after loading.

Microenvironment and cytoskeletal regulation

Mechanotransduction signals require cytoskeletal changes to transduce the mechanical response. In this regard, the microenvironment surrounding the cells modulates the mechanoreponse and has been investigated under hydrostatic pressure (Steward *et al.*, 2012; Steward *et al.*, 2013; Zhao *et al.*, 2015). Steward *et al.* (2012) compared the hydrostatic pressure response between cells encapsulated within agarose and fibrin hydrogels. Initially, MSCs within agarose have a rounded morphology due to no direct interactions, whilst in fibrin they adopt a fibroblastic morphology through integrin-receptor-

mediated connections. The differing interactions alter matrix elaboration during chondrogenesis whereby increased GAG synthesis is observed within agarose, whilst collagen synthesis is enhanced in fibrin. Upon application of hydrostatic pressure, only fibrin-encapsulated MSCs appear to increase GAG and collagen II synthesis. As previously discussed, this response is partially mediated by integrins, since their inhibition lead to loss in this beneficial effect under pressure stimulation (Steward *et al.*, 2012).

In a follow-up study, Steward *et al.* (2013) examined the effect of substrate or scaffold stiffness on pressure-stimulated MSC chondrogenesis. Under unloaded conditions, MSCs in stiffer hydrogels (4 %

Table 3b. Summary of the effects of hydrostatic pressure on pre-differentiated chondrogenic MSCs.

Publication	Cell source	Pellets/scaffold	Loading regime	Pre-differentiation period	Principal findings
Safshekan <i>et al.</i> , 2012	Human adipose MSCs	Pellet	Cyclic, 5 MPa, 0.5 Hz, 4 h/d, 7 d loading (\pm TGF- β)	4 d	SOX9 and COL2A1 expression increased in the presence of TGF- β and loading. ACAN expression increased with pressure alone, synergistic effect combined with TGF- β .
Karkhaneh <i>et al.</i> , 2014	Rabbit bone marrow MSCs	Scaffold [2×10^5 cells/scaffold on either polycaprolactone (PCL)/polyvinyl alcohol (PVA) or gelatine-PCL-PVA scaffolds]	Cyclic, 5 MPa, 0.5 Hz, 2 s/d, 7 d loading	7 and 21 d	COL2A1 expression increases upon loading; no change in SOX9 or ACAN expression. GAG synthesis increases under loading in PCL-PVA scaffolds and decreases on gelatine-PCL-PVA scaffolds.
Hosseini <i>et al.</i> , 2015	Human bone marrow and adipose MSCs	Scaffold (86,500 cells/cm ² , rubber silicone)	Cyclic, 3 MPa, 0.5 Hz, 1 h/d for 3 d combined with shear stress (0.2Pa, 1 Hz), no TGF- β	18 d	SOX9, ACAN and COL2A1 expression enhanced under combined loading in both MSC sub-types.
Li <i>et al.</i> , 2016	Rabbit cartilage progenitor cells, infrapetallar fat pad MSCs	Scaffold (alginate beads)	Cyclic, 5 MPa, 0.5 Hz, 4 h/d	7 d	SOX9, ACAN and COL2A1 expression upregulated in both cell types after 2 and 4 week loading – increased matrix synthesis.
Zhao <i>et al.</i> , 2016	Rat bone marrow MSCs	Scaffold [4×10^6 cells in 1.5 % (w/v) alginate beads]	Cyclic: 14-36 kPa, 0.25 Hz, 1 h/d. Static: 20 kPa, 7 d loading	7 d	SOX9 expression increases under loading, COLXA1 expression and matrix staining are reduced.

agarose) produce less extracellular matrix than in softer hydrogels (1 %). Application of hydrostatic pressure results in enhanced chondrogenic gene expression and matrix synthesis with increasing hydrogel stiffness. The authors investigated the changes in cytoskeletal elements under pressure, as alterations in these structures relate to cell morphology and shape that are influenced by the surrounding environment and stimuli. Indeed, previous studies have indicated that prevention of F-actin polymerisation induces chondrogenesis through development of a rounded cell shape (Daniels and Solursh, 1991; Daniels and Sandra, 1990; Woods *et al.*, 2005). Steward *et al.* (2013) demonstrated that within stiff hydrogels, more actin development is observed in unloaded controls, leading to a reduced chondrogenic response as compared to that found for cells in softer gels. Examination of vimentin (intermediate microfilament), tubulin (microtubule) and vinculin (focal adhesion molecule involved in anchorage to actin cytoskeleton) shows that vimentin staining become more diffuse upon loading application, whereas the other elements do not alter their structure (Fig. 2c). The authors suggested that this diffuse staining may be related to the process of depolymerisation due to pressurisation and that vimentin is more sensitive to hydrostatic pressure as compared with other cytoskeletal elements. siRNA

knockdown of vimentin within MSCs results in inhibited cartilage matrix production (Bobick *et al.*, 2010). Furthermore, vimentin interacts with actin and the integrins $\alpha v \beta_3$ and $\alpha_2 \beta_1$, indicating a potential mechanotransduction pathway that requires further elucidation (Steward *et al.*, 2013).

In relation to these phenomena, the development and presence of the pericellular matrix plays an important role in hydrostatic pressure mechanotransduction response and cytoskeletal behaviour. Application of the integrin inhibitor RGDS to stiff hydrogels in an unloaded situation results in more diffusive vimentin staining, leading to increased chondrogenic matrix production as compared with untreated controls (Steward *et al.*, 2013). However, in combination with hydrostatic pressure, vimentin do not alter its structure in the presence of RGDS, thus blocking the anabolic effects of hydrostatic pressure. This indicates the importance of both vimentin and upstream integrins and receptor channels to transduce the pressure-induced signal through the pericellular matrix.

In relation to the cytoskeletal changes, the role of GTPases has been investigated under hydrostatic pressure (Zhao *et al.*, 2015). GTPases perform an important role in cytoskeletal regulation within cells. RhoA *via* Rho-kinase (ROCK) plays a critical role in actin organisation and promotes a spindle-shaped

morphology in MSCs while inhibition of RhoA/ROCK signalling promotes chondrogenesis (Woods and Beier, 2006; Woods *et al.*, 2005). A member of the GTPase family that is involved in chondrogenesis is Rac1. Rac1 inhibition reduces MSC condensation during the early stages of chondrogenesis through related expression of cell-adhesion genes and proteins, N-cadherins. This subsequently leads to reduced chondrogenic gene expression and matrix production. In contrast, overexpression of Rac1 results in higher N-cadherin gene expression and subsequent chondrogenic gene expression, thus demonstrating its importance during the early stages of MSC cartilage differentiation (Woods *et al.*, 2007). Zhao *et al.* (2015) examined the role of RhoA and Rac1 in MSC chondrogenesis under the influence of hydrostatic pressure. In the presence of a RhoA agonist, the beneficial effects of pressure on chondrogenic gene expression and GAG deposition are inhibited. In contrast, in the presence of a RhoA inhibitor, hydrostatic pressure induces a significant increase in both gene expression and matrix formation (Fig. 2a). Use of a Rac1 inhibitor significantly inhibits anabolic response under pressure, whereas use of a Rac1 agonist enhances chondrogenesis. This indicates a primary role of Rac1 in pressure-induced chondrogenesis. The study did not investigate the relationship between Rac1 and cytoskeletal elements or upstream G-protein coupled receptors (GPCRs) involved in this anabolic response but it can be postulated that actin reorganisation through Rac1 and alterations in vimentin structure results in enhanced chondrogenesis under hydrostatic pressure.

Candidates for future analysis: membrane-bound receptors, ion channels, primary cilia and nuclei

The integrin family of receptors is involved in hydrostatic pressure chondrogenesis, both with respect to initial signal transduction and in its interaction with vimentin, although no specific integrin was investigated by Steward *et al.* (2013). An integrin expressed during MSC chondrogenesis is $\alpha_{10}\beta_1$. This is one of the principal collagen-binding receptors, alongside α_1 , α_2 and α_{11} . During MSC chondrogenesis, α_{10} increases in correlation with collagen II synthesis, whilst α_{11} decreased. Furthermore, application of basic fibroblastic growth factor 2 (FGF2) to MSCs in monolayer increases α_{10} expression and this, subsequently, upregulates collagen II gene expression during chondrogenesis (Lundgren-Akerlund and Aszodi, 2014). However, whether α_{10} is involved in mechanical stimulation, specifically under hydrostatic pressure, remains to be elucidated (Bengtsson *et al.*, 2005; Lundgren-Akerlund and Aszodi, 2014; Varas *et al.*, 2007). Furthermore, recent investigations have also implicated α_8 integrin in MSC chondrogenesis (LaPointe *et al.*, 2013). Thus, insights into specific integrins and how they are influenced under pressure is an area for future studies (Fig. 3a).

The plasmalemma contains ion channels that are potentially involved in mechanotransduction signalling cascade (reviewed by Lewis and Barrett-Jolley, 2015). One set of ion channels that has been studied are the transient receptor potential vanilloid (TRPV) channels (reviewed by Krupkova *et al.*, 2017).

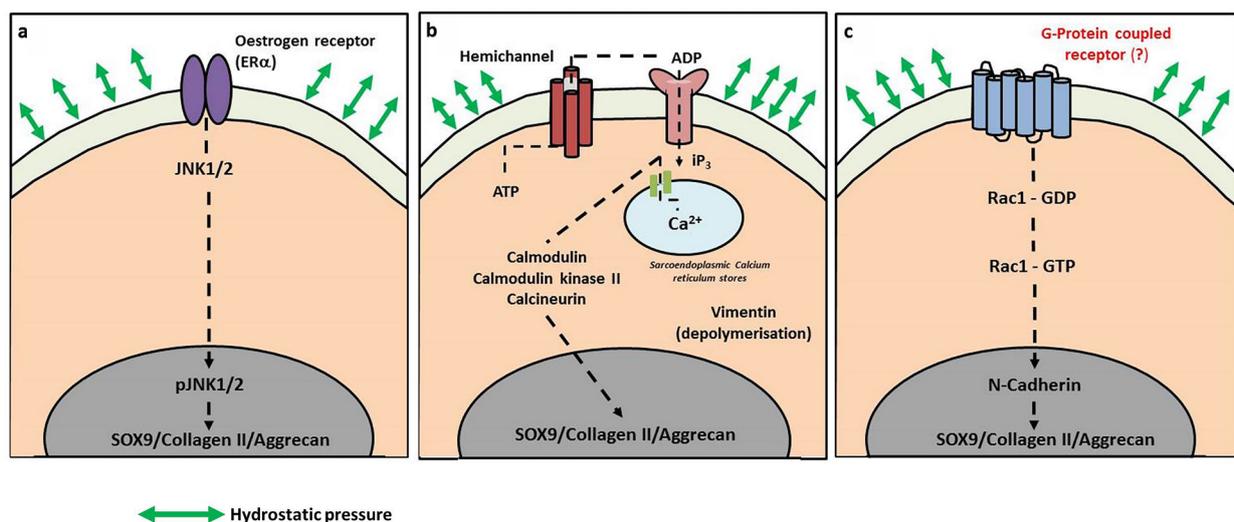


Fig. 2. Schematic diagrams summarising the pathways under the influence of hydrostatic pressure during MSC chondrogenesis that have been examined in previous publications. (a) Pressure upregulates oestrogen receptor in MSC chondrogenesis and the oestrogen receptor pathway exerts its anabolic response *via* JNK. Voltage-gated calcium ion channels and calcium stores (SERCs) are activated under hydrostatic loading *via* (b) purinergic signalling that stimulates calcium signalling *via* ATP release from hemichannels and interaction with cellular purine receptors, *e.g.* P2X and P2Y. Pressure-stimulated chondrogenesis influences the cytoskeletal structure *via* GTPases, specifically (c) Rac1-GTPase induces the anabolic response under pressure *via* activation of N-cadherins, with MSC condensations and subsequent chondrogenesis.

Murumatsu *et al.* (2007) demonstrated that transient receptor potential cation channel subfamily V member 4 (TRPV4) directly influences chondrogenic genes *via* SOX9 expression, as use of a TRPV4 antagonist reduces SOX9 expression and prevents MSC chondrogenesis. In addition, the authors demonstrated the importance of calcium signalling in TRPV4 activation, as use of either a calcium chelator or calmodulin inhibitor prevents SOX9 expression and subsequent chondrogenesis (Murumatsu *et al.*, 2007). In articular-cartilage chondrocytes, stimulation of TRPV4 under hypoosmotic stress increases chondrogenic gene expression and matrix accumulation (GAG and collagen content). However, the presence of a TRPV4 inhibitor (GSK205) reduces both osmotic-stressed-induced matrix accumulation and calcium signalling (O'Connor *et al.*, 2014; Phan *et al.*, 2009). Interestingly, an interaction between $\alpha_1\beta_1$ integrin and TRPV4 has been demonstrated under osmotic stress in chondrocytes, whereby loss of

the integrin prevents transduction of signalling *via* TRPV4 (Jablonski *et al.*, 2014). Furthermore, TRPV4 knock-out mouse models show that despite reduced severity in ageing-associated OA, TRPV4 deficiency still results in premature OA and, thus, its presence is critical for cartilage homeostasis, whilst also being a potential OA marker (Clark *et al.*, 2010; Lamande *et al.*, 2011; O'Connor *et al.*, 2016). In the context of hydrostatic pressure, due to the force resulting from fluid pressurisation being applied, there is an osmotic stress exerted on cells that varies between hypo and hyperosmotic stress, especially under cyclic hydrostatic pressure. How TRPV4 and other TRP channels are involved under pressure remains an area to be investigated (Fig. 3b).

A further set of receptors that may be under the influence of hydrostatic pressure are the GPCRs. GPCRs are stimulated under mechanical loading, although it is unknown whether they are involved in hydrostatic pressure mechanotransduction (reviewed

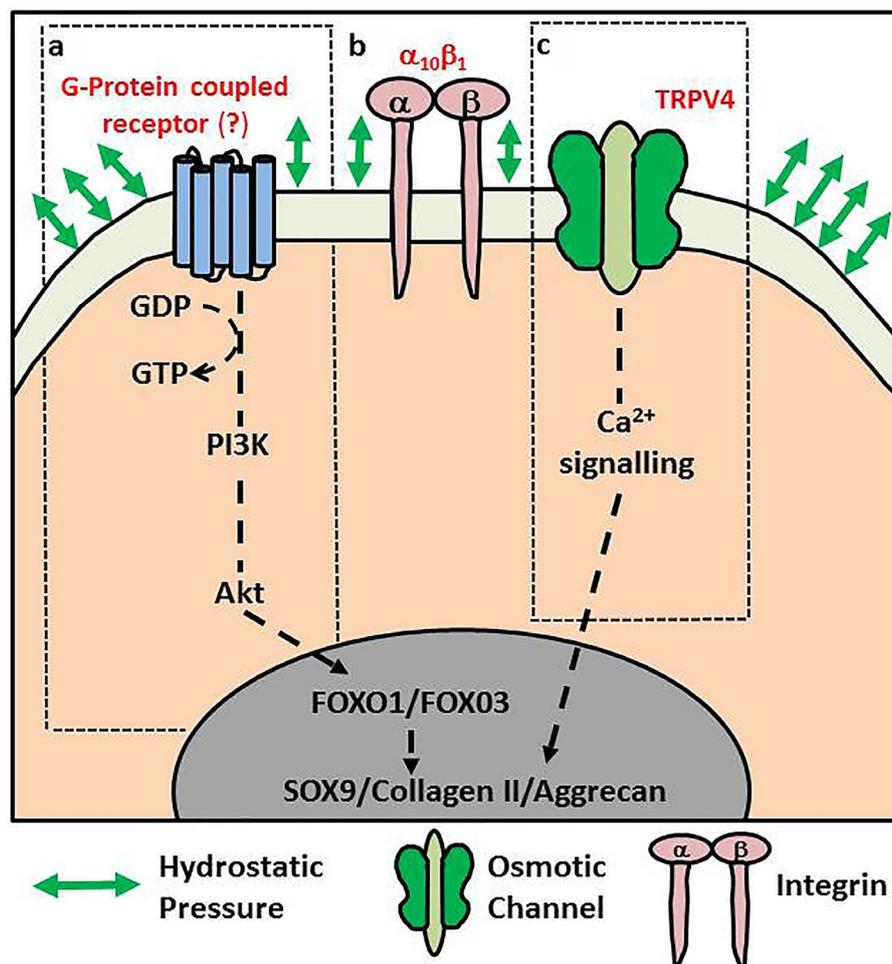


Fig. 3. Schematic diagram showing hypothetical receptors and channels that could be under the influence of hydrostatic pressure. (a) Integrin $\alpha_{10}\beta_1$ increase in expression during MSC chondrogenesis and its stimulation under pressure have yet to be elucidated. (b) Due to hydrostatic pressure involving fluid pressurisation, osmotic channels may be implicated in the process; (c) specifically, TRPV4 is a potential membrane channel stimulated by hydrostatic pressure during MSC chondrogenesis, exerting its response *via* downstream calcium signalling. The G-protein-coupled receptors are stimulated under mechanical load, although not directly implicated under hydrostatic pressure. The downstream PI3K/Akt/FOXO pathway is potentially activated, thus, preventing chondrogenic hypertrophy under these conditions.

by Shah *et al.*, 2014). Studies have described the prevention of hypertrophy under pressure, although the pathways influencing this outcome were not demonstrated. Interestingly, studies have described the activation of the downstream GPCR pathway, phosphoinositide 3-kinase/Akt/forkhead box protein O (PI3K/Akt/FOXO). This signalling mechanism is involved in preventing hypertrophy in MSC chondrogenesis (Lee *et al.*, 2013). Furthermore, recent investigations implicated the FOXO transcription factors in preventing OA-associated cartilage damage (Akasaki *et al.*, 2014; Matsuzaki *et al.*, 2018). Whether this downstream GPCR pathway and its respective receptor is stimulated under hydrostatic pressure is not known (Fig. 3c).

Recent studies in articular-cartilage chondrocytes have also begun to investigate the role of the primary

cilium in mechanotransduction (Bodle and Lobo, 2016; Knight *et al.*, 2009; McGlashan *et al.*, 2006; Phan *et al.*, 2009; Wann *et al.*, 2012). The cilium is a membrane-coated axoneme that emanates from the cell surface into the cell microenvironment and its loss leads to skeletal abnormalities (Bodle and Lobo, 2016). Under compressive loading, the cilium is involved in the activation of ATP-induced calcium signalling in articular cartilage chondrocytes, although it is not the initial mechanoresponder during this process (Wann *et al.*, 2012). In relation to potential hydrostatic pressure-stimulated channels, TRPV4 localises on the cilium and cell membrane of porcine chondrocytes. Interestingly, TRPV4-mediated calcium signalling requires the presence of an intact cilium, even in the presence of a TRPV4 activator and hypo-osmotic loading (Phan *et al.*, 2009).

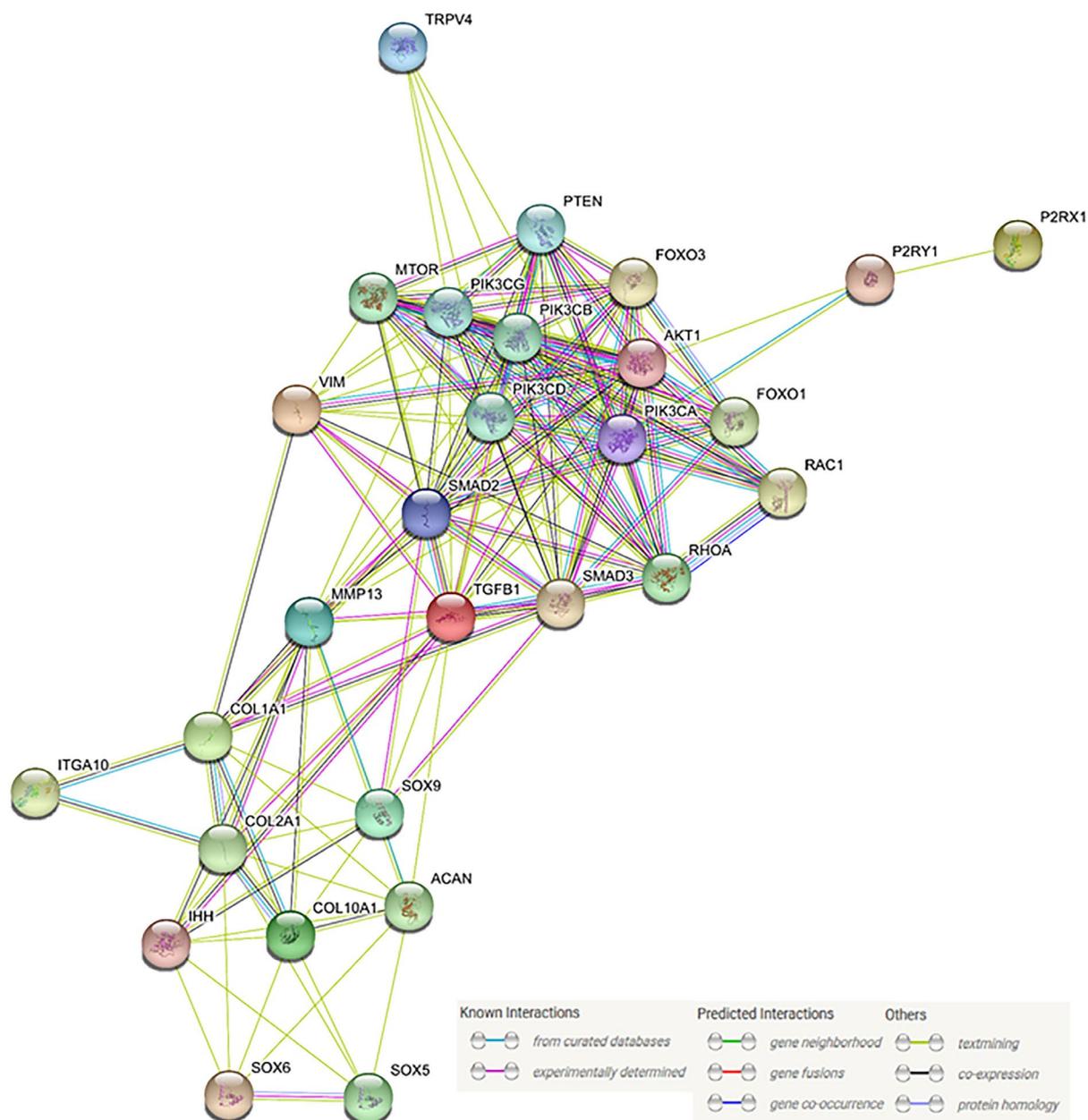


Fig. 4. STRING database analysis based on genes and proteins under the influence of hydrostatic pressure and chondrogenesis, showing interactions among proteins based on database analysis.

Integrins (α_2 , α_3 and β_1) and connexins are also found on chondrocyte cilia (Knight *et al.*, 2009; McGlashan *et al.*, 2006). Thus, rather than the membrane-bound receptors and ion channels, the cilium may be the initial mechano-sensor for certain types of loading, potentially hydrostatic pressure. However, since the primary cilium can act as a pressure sensor under compressive and tensile loading, it is unlikely to be an influence under hydrostatic pressure due to minimal fluid flow that prevents bending of the cilium (Bell, 2008). Based on the present review, it may also be hypothesised that due to the presence of the receptors and ion channels, the cilium may be indirectly activated and act as an initial mechanosensor under hydrostatic pressure. No studies have described the involvement of the cilium in pressure-induced MSC chondrogenesis; therefore, further elucidation is required.

Researchers have also begun to examine the nucleus and its roles during mechanical loading. Studies have begun to show that the nucleus acts as a mechanosensor upon stimulation, under compressive and tensile loading (Heo *et al.*, 2018; Martins *et al.*, 2012). However, whether the nucleus can act as a mechanosensor under hydrostatic pressure when there is no direct cellular deformation needs to be established.

Discussion

Hydrostatic pressure has an anabolic effect on MSC chondrogenesis, whereby it increases cartilage gene expression (*SOX9*, *COL2A1*, *ACAN*) and matrix formation (GAG and collagen II). However, there

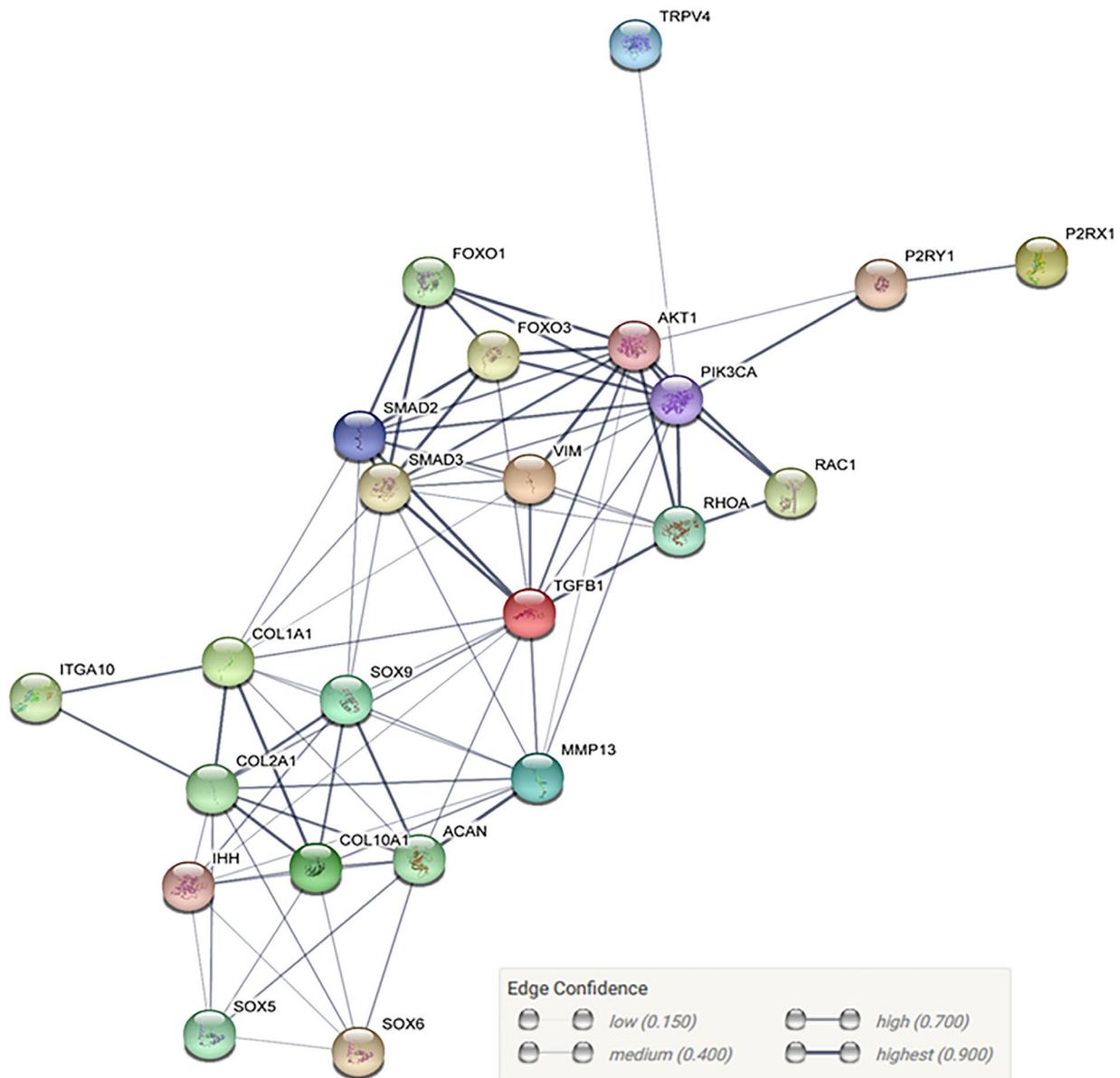


Fig. 5. STRING database analysis based on genes and proteins under the influence of hydrostatic pressure and chondrogenesis, showing the probability of protein interactions.

remain open questions that need to be addressed in future investigations.

The optimal loading conditions for anabolism remain unclear due to differences in loading parameters (*i.e.* peak pressure, frequency) among studies. Furthermore, the timing and length of loading under pressure also differ. Meyer *et al.* (2011) demonstrated that pressure has a larger effect when applied continuously as compared to loading from day 21 onwards within a cell-scaffold model. This potentially indicates that the embryonic and mesenchymal stages are the points of chondrogenesis under the greatest influence of pressure, corresponding to the theoretical models and early publications on hydrostatic pressure-induced chondrogenesis (Angele *et al.*, 2003; Ogawa *et al.*, 2009). However, there remains an open question regarding the timing of loading or length of preconditioning. Furthermore, expression of endogenous TGF- β and other pro-chondrogenic factors by MSCs under hydrostatic pressure, with or without pre-stimulation, is incompletely studied. It may be hypothesised that the length of preconditioning could influence this latter phenomenon.

Based on theoretical models, hydrostatic pressure has been shown to stabilise the chondrocyte phenotype and, thus, prevent hypertrophy. However, publications have described both prevention and enhancement of hypertrophy gene expression (collagen X and MMP-13) under hydrostatic pressure. One method to prevent hypertrophy is physioxic culture. *In vivo*, cartilage resides under a low-oxygen condition between 2-5 % oxygen or physioxia (Brighton and Heppenstall, 1971; Lafont, 2010; Lund-Olesen, 1970) and recent investigations have shown that hypertrophy may be prevented under physioxia (Adesida *et al.*, 2012; Anderson *et al.*, 2016; Lee *et al.*, 2013). Combining physioxia and pressure may be a solution for preventing hypertrophy during MSC chondrogenesis.

A variety of mechanisms for mechanotransduction under hydrostatic pressure have been explored, particularly those associated with purinergic and calcium signalling. However, the receptors and channels involved in transducing the signal need to be established. This could uncover previously unknown pathways involved in transduction of hydrostatic pressure in chondrogenic MSCs. To date, TRPV4, $\alpha_{10}\beta_1$ integrin and GPCRs are candidate receptors that may be activated under hydrostatic pressure (Fig. 3) (Bengtsson *et al.*, 2005; Lee *et al.*, 2013; O'Connor *et al.*, 2014; Phan *et al.*, 2009; Shah *et al.*, 2014; Varas *et al.*, 2007).

To develop an understanding of the interactions that could be under the influence of pressure, Szklarczyk *et al.* (2015) performed a STRING analysis based on the literature and hypothesising that membrane receptors and channels may be involved in hydrostatic pressure chondrogenesis (Fig. 4). The analysis shows the relationships among proteins based on experimental and theoretical results compiled and

evaluated from various databases and predicts the probability of interactions among them. Fig. 4 shows the interactions based on experiments and curated databases, whilst also checking for evidence from text mining. The analysis indicates a major interaction among PI3K/FoxO, TGF- β /SMAD and GTPases (RhoA and Rac1), whilst the associated chondrogenic genes are grouped together. Probability analysis of these interactions in the described pathways shows high correlations among the described downstream pathways and chondrogenic genes (Fig. 5). Purinergic signalling receptors, P2YR1 and P2XR2, demonstrate a strong interaction with respect to PI3K pathway, which is expected due to P2Y receptors interaction with GPCRs; although, studies performed under hydrostatic pressure have not directly examined this link. Furthermore, the STRING assessment also indicates no evidence for direct interactions between the PI3K pathway, chondrogenesis genes or upstream ion channels, *e.g.* TRPV4. Most interactions between pathways and chondrogenic genes involve TGF- β , as indicated by its centrality in both networks. This is not surprising since TGF- β is a growth factor that stimulates chondrogenesis. However, the analysis shows that there are many targets described in the literature that require further investigation in the context of hydrostatic pressure.

In summary, hydrostatic pressure has a positive influence on MSC chondrogenesis, increasing both genes and matrix proteins. However, there remains conjecture on the timing at which it induces the most beneficial response and whether it prevents hypertrophy. The underlying mechanisms controlling the hydrostatic pressure response remain to be elucidated and may offer new targets for therapies if novel pathways that exerts its positive effects are found.

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Discussion with Reviewers

Farshid Guilak: MSCs respond to various mechanical stimuli. Can the authors comment on their relative contribution?

Authors: Researchers have begun to examine the relative contribution of different physical stimuli (compressive, tensile, shear and hydrostatic pressure) to MSC chondrogenesis using both experimental and computational models. Schatti *et al.* (2011) showed that uniaxial compression and shear alone is insufficient to induce MSC chondrogenesis but that combined compression and shear are required for chondrogenic induction. Finite element analysis shows that compressive deformation of an MSC-seeded fibrin-polyurethane scaffold resulting from combined cyclic compression and shear loading is the principal stimulus that induces MSC chondrogenesis under load (Zahedmanesh *et al.*, 2014, additional reference). However, as noted by the authors and in relation to the present review, the high scaffold porosity results in negligible involvement of hydrostatic pressure due to water being released from the scaffold upon compression. However, MSCs, seeded in scaffolds with a lower porosity (*e.g.* agarose) that mimic native articular cartilage, demonstrate chondrogenic induction under uniaxial compressive loading due to the presence of both compressive and hydrostatic pressure. The latter stimulus is present due to a reduction in fluid loss within the system. In contrast to the previously described model, chondrogenesis occurs in areas of maximum hydrostatic pressure

(Kisiday *et al.*, 2009, additional reference). Thus, further *in vitro* and computational modelling studies are required to delineate the relative contribution of these different stimuli to MSC chondrogenesis, especially in multiaxial loading systems.

Daniel Kelly: *In vivo*, implanted cells will be subjected to high levels of hydrostatic pressure. Based on the review of the literature, have the authors found any evidence to suggest that MSCs require pre-culture to respond positively to hydrostatic pressure (in terms of either increases in matrix deposition or suppression of hypertrophy) or should MSCs be able to respond positively to the hydrostatic pressure they experience *in vivo* without pre-culture?

Authors: To the authors' knowledge, there is no understanding about whether MSCs react positively to *in vivo* hydrostatic pressure with or without pre-culture. No published studies describe implantation of hydrostatic-pressure-stimulated chondrogenic MSCs into animal models. This is clearly an area in need of testing.

Daniel Kelly: Is there any evidence suggesting that the beneficial effects of hydrostatic pressure on chondrogenesis can be mimicked by specific drugs?

Authors: The authors are unaware of specific drugs that mimic the beneficial effects of hydrostatic pressure on MSC chondrogenesis. However, it is possible that the secreted factors (including paracrine factors, exosomes and microvesicles) released from MSCs during pressure stimulation may be exploited in this manner. Recent publications have addressed the importance of the secreted factors released during mechanical stimulation in MSC chondrogenesis, particularly in dynamic loading systems (compression and shear) conditions (Fahy *et al.*, 2018; Gardner *et al.*, 2016a; Phelps *et al.*, 2018; Vonk *et al.*, 2018, additional references). Further investigations into the MSC chondrogenic secretome under hydrostatic pressure could potentially lead to the development of drugs that utilise these factors. Furthermore, drugs that target specific pathways that are known to be involved in the pressure-induced anabolic response would also be exploitable for drug development.

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Editor's note: The Scientific Editor responsible for this paper was Martin Stoddart.