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Review Article

Wnt3a and wnt5a as Potential Chondrogenic Stimulators for Nucleus Pulposus Cell Induction: A Comprehensive Review

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Low back pain remains a highly prevalent pathology engendering a tremendous socioeconomic burden. Low back pain is generally associated with intervertebral disc (IVD) degeneration, a process involving the deterioration of nucleus pulpous (NP) cells and IVD matrix. Scientific interest has directed efforts to restoring cell numbers as a strategy to enable IVD regeneration. Currently, mesenchymal stromal cells (MSCs) are being explored as cell therapy agents, due to their easy accessibility and differentiation potential. For enhancement of MSCs, growth factor supplementation is commonly applied to induce differentiation towards a chondrogenic (NP) cell phenotype. The wnt signaling pathways play a crucial role in chondrogenesis, nonetheless, literature appears to present controversies with regard to wnt3a and wnt5a for the induction of NP cells, chondrocytes, and MSCs. This review aims to summarize the reporting on wnt3a/wnt5a mediated NP cell differentiation, and to elucidate the mechanisms involved in wnt3a and wnt5a mediated chondrogenesis for potential application as cell therapy supplements for IVD regeneration. Our review suggests that wnt3a, subsequently replaced with a chondrogenic stimulating growth factor, can enhance the chondrogenic potential of MSCs *in vitro*. Contrariwise, wnt5a is suggested to play a role in maintaining cell potency of differentiated NP or chondrogenic cells.

Keywords: Wnt, Chondrogenesis, Mesenchymal stem cells, Intervertebral disc, Nucleus pulposus, Regeneration

INTRODUCTION

Low back pain (LBP) is a disorder presenting itself among all adult-ages[1] affecting an estimated 632 million people globally.[2] Contemporary treatments are primarily focused on pain relief with a lack of therapeutic strategies able to target the pathogenesis of LBP. A particular shortcoming, considering up to 15% LBP-patients do not respond to conservative treatment intervention and progress to chronic LBP.[3] The onset of LBP is generally associated with the progression of intervertebral disc (IVD) degeneration, a complex process involving a reduction in active cell numbers, tissue disorganization, and overall loss of the IVDs biomechanical features. Particularly, the loss of proteoglycans from the central nucleus pulposus (NP) and consequential deterioration of water-retention, limits the ability of the IVD to distribute complex loads on the spine.[4] In order to alleviate IVD degeneration-associated LBP, restoration of the IVD and its biomechanical features will prove crucial. Nevertheless, similar to other cartilage-based tissues, the IVD presents a challenging tissue to regenerate.[5] Due to the avascular nature of the IVD, the *in situ* environment presents a nutrition-poor, biochemically harsh environment that is restricted in endemic cell attraction, and overall dependent on relatively low cell numbers for matrix production.[6]

Considering the reduction in already low cell numbers is a hallmark for IVD degeneration, emphasis has been placed on methods to restore NP cell numbers as a strategy to enable IVD regeneration. Cell-based therapies are increasing in number and are currently being evaluated in multiple human clinical trials.[7] Nevertheless, an optimal cell type or preconditioning method to enable optimized regeneration of NP tissue has not yet been established.[8-10]

Wnt signaling holds a key role in joint development, homeostasis, and pathogenesis. The prominence of wnt signaling in cartilage tissues was initially discovered in chicken limb cartilage.[11,12] Both the activation and inhibition of wnt signaling result in cartilage breakdown.[13] To support cartilage maintenance, careful regulation of wnt is therefore required. However, the exact regulatory mechanisms involved in wnt-mediated cartilage homeostasis are not yet established.[14] The diverse wnt family, consisting of 19 members in homo sapiens,[15] utilizes different signaling pathways and cell functions. Wnt11 and especially wnt5a are reported to be beneficial for chondrogenesis, however literature predominantly assesses their function within embryonic development.[16] On the contrary, Wnt7a and wnt14 are reported to inhibit or reverse chondrogenesis.[17,18] Wnt3a, however, is particularly of interest due to numerous contradictions in the literature.[19,20] Most studies tend to agree upon the positive regulatory effect of wnt3a on cell proliferation,[16,21,22] however, both wnt3a activation and inhibition have been reported to downregulate cartilage phenotypic markers, and reduce glycosaminoglycan (GAG) production.[13,21] Interestingly, in combination with other growth factors; e.g., fibroblast growth factor (FGF),[22] transforming growth factor (TGF)-β,[23,24] and bone morphogenetic protein (BMP)[25,26] family, wnt3a appears to be beneficial for MSCs to adopt a chondrogenic phenotype in 2-dimensional, and 3-dimensional cultures.[23,27] Whether wnt3a and wnt5a are in fact beneficial for chondrogenesis remains arguable, however, their presence in cartilage-based tissues is undisputable, raising interest on the potential induction capacity of wnt3a/wnt5a on the cartilage-based NP. Therefore, it is considered valuable to reach a consensus on wnt3a and wnt5a in chondrogenesis for regeneration of the IVD. This review aims to assess the trend in effects of wnt3a and wnt5a on cells with chondrogenic potency, particularly the response in potency, proliferation and induction of chondrogenic differentiation. Knowledge regarding the potential of wnt3a and wnt5a to stimulate NP cell induction could potentially provide new insights for (cell-) therapies enhancing IVD regeneration.

Wnt SIGNALING IN INTERVERTEBRAL DISC DEVELOPMENT

The 19 identified wnt ligands and their corresponding receptors[15] are pivotal for cellular development, function, and tissue homeostasis, in various tissues, including cartilage tissues[11,12] such as the IVD.[28,29] Specifically during early development, IVD cells are highly dependent on wnt signaling for appropriate development.[28,29] During these early stages, high activity of wnt is predominantly observed in the annulus fibrosis (AF) and the endplate (EP), however, with maturation, the AF and EP present weakening in signaling activity, while wnt activity persists in the NP even at later stages of development.[28] The importance is underlined by *in vivo* experiments presenting induction of IVD degeneration by suppression of wnt signaling in mice.[30] Similarly, *in vitro* work demonstrated that both induction and interference of wnt induce articular chondrocyte differentiation,[13] and wnt activation in NP cells may result in cell senescence.[31,32] In short, a balance of wnt signaling is crucial for maintaining cartilage homeostasis. Nevertheless, the exact regulatory mechanisms involved in wnt-mediated homeostasis remains poorly understood. This is partly due to the large number of receptor-types involved in reacting to wnt.[33] This process is further complicated by the involvement of two distinctively separate downstream pathways effectuated to establish a cellular response; i.e., canonical and noncanonical signaling.

CANONICAL Wnt SIGNALING

Wnt proteins can bind to the receptor complex consisting of Frizzled and the low-density lipoprotein receptor-related protein (LRP) 5/6, provided that both are expressed on the surface, forming a trimeric complex.[34] Activation leads to the recruitment of Dishevelled (Dsh) and Axin. Subsequently, the formed protein complex consisting of Axin, Adenomatous *Polyposis Coli*, and glycogen synthase kinase-3β (GSK-3) is not capable to partake in proteasome activation. As together with casein kinase 1α, this complex is responsible for the ubiquitination of β-catenin triggering its degeneration by proteasomes.[35] Absence of this complex disables degradation of β-catenin and instead accumulates and translocates to the nucleus. Here, it interacts with the transcription factors lymphoid enhancer binding factor/T-cell specific transcription factor, activating transcription of a variety of *WNT* target genes (Fig. 1).[36]

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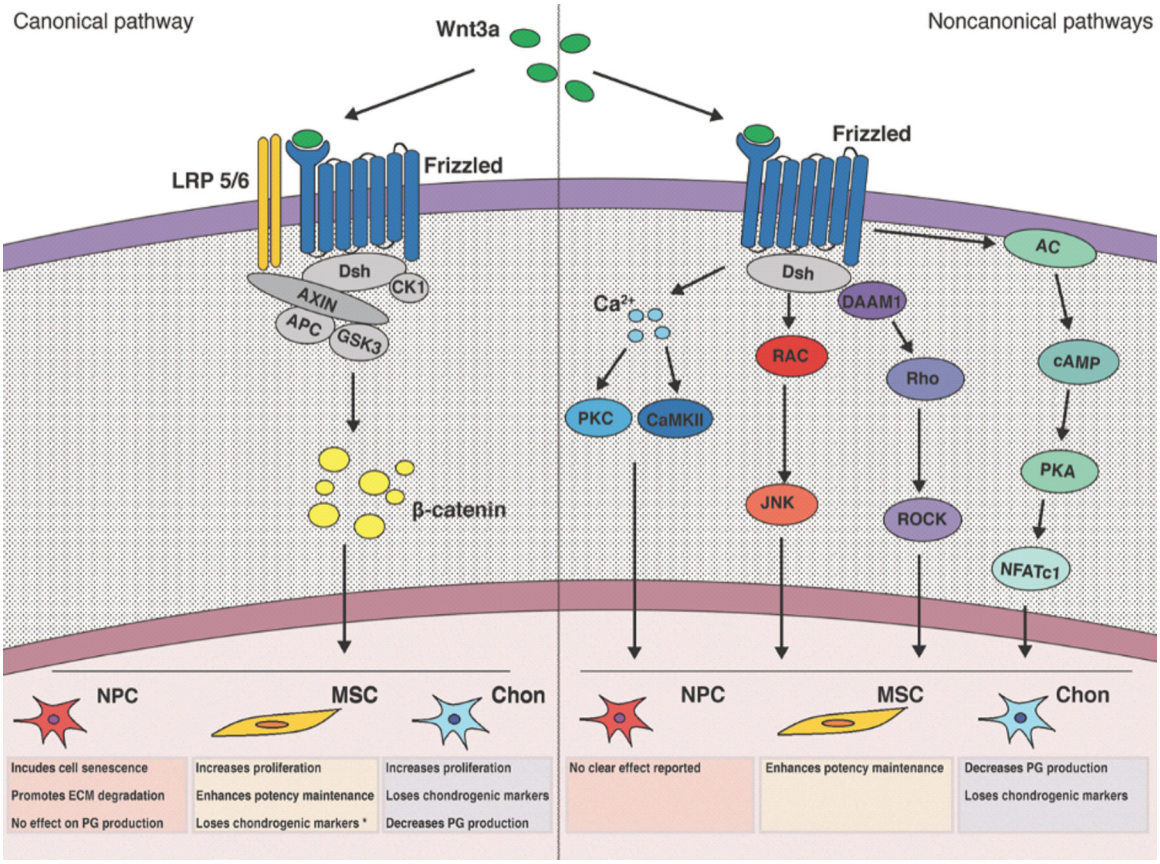

Fig. 1. Canonical and noncanonical signaling options initiated via wnt3a and the concluded outcome. Wnt3a can bind to Frizzled and low-density lipoprotein receptor-related protein (LRP) 5/6 which leads to the recruitment of Dishevelled (Dsh) and Axin. The formation of the complex of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK3) is prevented and thus β-catenin is not degraded by this complex (with the help of casein kinase 1α [CK1]) which is canonical signaling. With noncanonical signaling, wnt3a binds to Frizzled without LRP 5/6. Activated Frizzled can lead to the activation of calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC). Dsh is also able to activate Rac which in turn induces c-Jun N-terminal kinase (JNK). Moreover, Dsh can interact with DAAM1 to activate Rho which in turn activates ROCK. The last noncanonical pathway is through activation of adenylate cyclase (AC), which triggers cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) inhibiting the transcription factor NFATc1. This figure is a hypothetical estimation based on the literature discussed in this paper. Chon, chondrocytes; NPC, nucleus progenitor cell; MSC, mesenchymal stromal cell; PG, proteoglycan.

NONCANONICAL Wnt SIGNALING

The noncanonical signaling pathway is poorly understood compared to the well-studied canonical pathway, partly due to multiple agents and mechanisms being involved.[37] Where the canonical pathway has only a few key players, mainly involved in degradation of β-catenin, thus preventing interaction in the nucleus for transcription, the noncanonical pathway has multiple agents capable of initiating various subsequent processes which are relatively difficult to separate and study. In contrast to canonical signaling, noncanonical signaling is β-catenin independent and while wnt still requires binding to Frizzled, the coreceptor LRP 5/6 is not involved.[38] Interaction with Frizzled can lead to the activation of calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC),[38] referred to as the wnt/calcium pathway. Noteworthy is that this pathway is found to inhibit canonical signaling.[39] Additionally, in the planar cell polarity (PCP) pathway, Dsh is activated which regulates a variety of signaling cascades, for example activation of Rac, which in turn activates c-Jun N-terminal kinase (JNK). Additionally, Dsh activates Rho through Daam1 activating e.g., Rho-kinase and Profilin.[39] These processes are suggested to play a crucial role in cytoskeletal organization and cell adhesion as these are linked to actin polymerization.[39] Lastly, in the wnt/

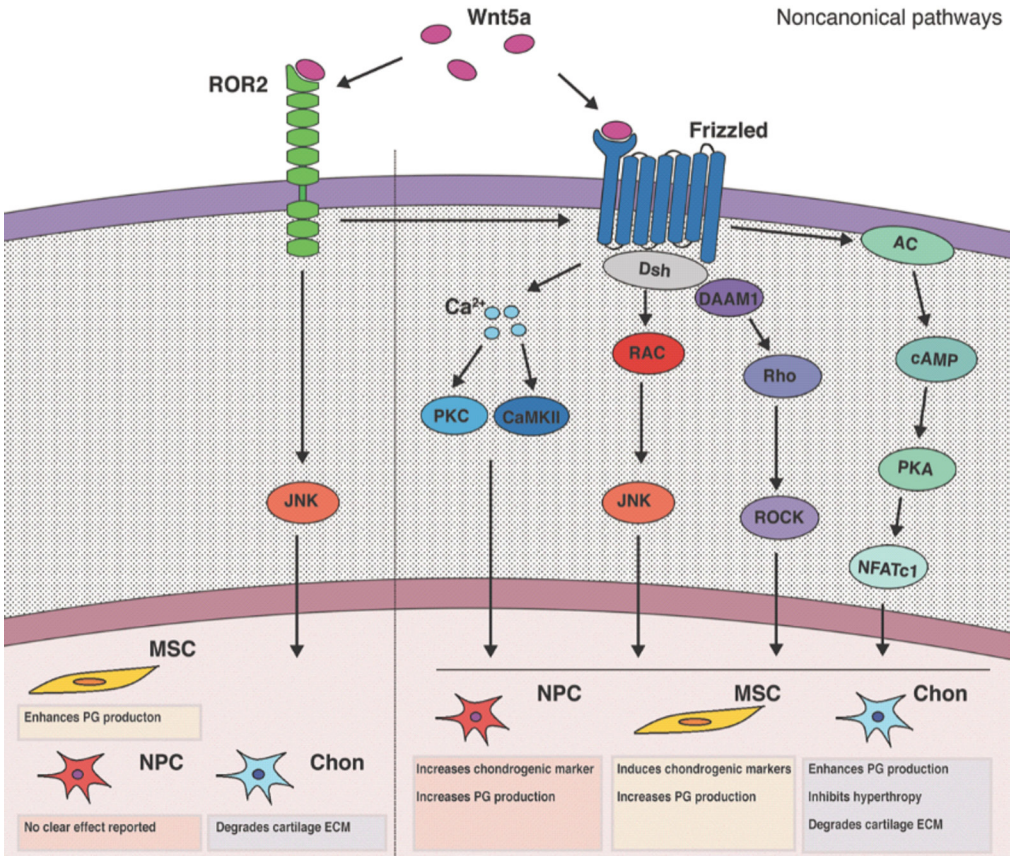

Fig. 2. Signaling options initiated via wnt5a and the concluded outcome. Wnt5a can bind to receptor tyrosine kinase-like orphan receptor 2 (ROR2) which leads to the activation of c-Jun N-terminal kinase (JNK). ROR2 is also able to act as a coreceptor with Frizzled. Wnt5a can bind to Frizzled and ROR2 leading to the activation of calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC). Dishevelled (Dsh) is also able to activate Rac which in turn induces JNK. Moreover, Dsh can interact with DAAM1 to activate Rho which in turn activates ROCK. The last pathway is through activation of adenylate cyclase (AC), which triggers cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) inhibiting the transcription factor NFATc1. This figure is a hypothetical estimation based on the literature discussed in this paper. Chon, chondrocytes; NPC, nucleus progenitor cell; MSC, mesenchymal stromal cell; PG, proteoglycan.

protein kinase A (PKA) pathway, Frizzled activates adenylate cyclase, which triggers cyclic adenosine monophosphate/PKA inhibiting the transcription factor NFATc1 (Figs. 1, 2).[40,41] Aside from Frizzled, wnts are shown to activate receptor tyrosine kinase-like orphan receptor 2 (ROR2), with wnt5a as their primary ligand.[42] Upon binding, ROR2 mediates activation of JNK which is able to inhibit β-catenin, thus interfering in canonical signaling. ROR2 can also function as a coreceptor together with Frizzled, in the absence of LRP 5/6, and as such activate Dsh,[43] leading to crosstalk with the PCP and wnt/calcium pathway (Fig. 2). Possible crosstalk within noncanonical signaling and canonical signaling, together with the many agents involved, causes the exact mechanisms surrounding these pathways to remain ambiguous.

Wnt LIGANDS IN CHONDROGENESIS

In terms of chondrogenesis, wnts can be described on the ability to regulate chondrogenic (de-)differentiation or hypertrophy. Most wnts have been studied thoroughly and are suggested to have clear effects on chondrogenesis. For example, wnt1, wnt4, wnt7a, wnt8, and wnt9a have been determined to inhibit chondrogenic differentiation.[19,20] Wnt5a and wnt5b however, have been reported to induce chondrogenesis in developmental stages, whereas it inhibits chondrogenesis in later stages.[19,20] The different effects during the various stages of development, causes the use of wnt5a and wnt5b in chondrogenic differentiation to remain ambiguous. Additionally, discrepancies in the literature on the chondrogenic effects of wnt3a, lead to

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reviews reporting contradictory conclusions, stating that wnt3a stimulates,[20] but also inhibits chondrogenic differentiation.[19] Another unusual characteristic of wnt3a is the capacity to activate both the canonical and noncanonical pathway[21,44] which might partly help explain the contradictory reporting on wnt3a effects. This theory is supported by studies reporting that downregulation of canonical signaling by e.g., dickkopf-related protein 1 (DKK1) as LRP antagonist, is able to partly rescue wnt3a-induced loss of chondrogenesis.[45] Similarly by applying a GSK-3 inhibitor could alleviate wnt3a repression of chondrogenesis, and was found to effectuate its regulation via β-catenin pathway.[46] Furthermore, decreased proteoglycan production caused by wnt3a could be recovered after addition of DKK3.[47] Recently, a knockdown of exotosin-1 (Ext1), encoding a glycosyltranserase required for heparin sulfate (HS) chain elongation in HS-proteoglycan biosynthesis, was determined to downregulate canonical wnt signaling activation but upregulate markers for chondrogenesis.[48] The collective data appears to suggest that canonical signaling is responsible for wnt3a mediated inhibition of chondrogenesis. However, separate wnt3a studies that blocked noncanonical signaling by specific inhibition of the Ca2+/CaMKII pathway, were likewise able to rescue wnt3a induced loss of the chondrocyte phenotype.[13,21] Expression of *COL2A1*, *aggrecan*, and *SOX9* all improved after Ca2+/CaMKII inhibition at higher rates than DKK1-mediated inhibition of canonical signaling.[13,21] Besides the CaMKII pathway, the PCP pathway has also been demonstrated to mediate dedifferentiation in chondrocytes. JNK inhibition resulted in the rescue of wnt3a downregulated chondrogenic markers SOX9 and COL2A1.[49] Therefore, the distinct effects on chondrogenesis by wnt3a cannot directly be related to the pathway that is activated. However, the balance of canonical and noncanonical signaling appears essential as both stimulation and inhibition of one specific pathway appear disadvantageous for chondrogenesis.

Wnt3a HAS A CELL TYPE DEPENDENT INFLUENCE ON CHONDROGENESIS WHEREAS THE INFLUENCE OF Wnt5a APPEARS HOMOGENOUS

Although wnt3a is capable of signaling through both canonical and noncanonical signaling,[21,44] the contradictory effects on chondrogenesis of wnt3a appears not to be dependent on which specific pathway is activated (Fig. 1). Therefore, the next question was whether the role of wnt3a in chondrogenesis is cell type dependent. Our findings (Table 1) confirm the ability of wnt3a to both induce and inhibit chondrogenesis, and indicates a cell type-specific response. In contrast, the response to wnt5a on chondrogenesis appears more homogeneous regarding cell types and supplementation methods (Table 1). Next, we review the differences in outcomes between cell types in response to being subjected to either wnt3a or wnt5a.

NUCLEUS PULPOSUS CELLS

NP cells subjected to an abundance of *WNT3A* via (lentiviral-mediated) overexpression[16,31,32] or wnt3a supplementation via medium[31] are not affected in chondrogenic marker expression nor proteoglycan production. However, proliferation was shown to be enhanced with overexpression of *WNT3A* in human NP cells relative to *WNT5a/WNT11* overexpression and non-induced NP cells.[16] Solely upregulation of wnt/β-catenin signaling caused no proliferation in rat-derived NP cells however, cell viability was determined to decrease with 50%.[32] Moreover, wnt/β-catenin signaling was demonstrated to regulate tumor necrosis factor (TNF)-α,[47] linking it to IVD degeneration,[50,51] suggesting once more that the balance of canonical and noncanonical signaling is crucial for the outcome of wnt3a treatment. Interestingly, upregulation of the PKC pathway, of the noncanonical signaling pathway, induced cell proliferation in rat NP cells.[52] However, this phenomenon has not been studied in human NP cells. Studies conducted on human cells primarily focus on the canonical pathway,[53] in which contradictive results are obtained as specific downregulation of wnt/β-catenin signaling leads to NP cell apoptosis,[54] while activation of wnt/β-catenin signaling is also reported to result in NP cell apoptosis.[55] Canonical and noncanonical signaling appears to have crosstalk leading to a chain of reactions which are likely challenging to control. One hypothesis is that the balance of canonical and noncanonical signaling is essential for inducing NP cell differentiation, thus making it challenging to regulate wnt3a with a specific inhibitor or stimulator of either canonical or noncanonical signaling. Adding to the complexity of wnt3a, other agents such as BMP2 and TGF-β are found to activate wnt/β-catenin[56] signaling whereas overexpression of R-Smad, an intracellular signaling protein downstream of the BMP/TGF signaling pathway, inhibits wnt/β-catenin signaling.[31] Crosstalk of the BMP/TGF-β family with wnt signaling is hypothesized to potentially control the balance of canonical and noncanonical signaling utilizing downstream signaling proteins like Smad.[31]

Overexpression of *WNT5A* in human NP cells results, in contrast to *WNT3A*, in the reduction of proliferation compared to

Table 1. Identified papers reporting the chondrogenic effect of wnt3a and wnt5a separated based on cell type

Cell type	Author	Year	Wnt	Addition during expansion	Addition during differentiation	Species	Culture	Supplementation method wnt3a	Signaling pathway	Proliferation	Cell potency	Chondrogenesis	Outcome summary
Mesenchymal stromal cells	Narcisi	2015	Wnt3a	FGF2, wnt3a	TGF-β1	Human	2D	In medium	Not confirmed	Increased	Enhanced	N/A	Increase of PG production, chondrogenic markers and proliferation
				Wnt3a	-					Increased	Neutral	N/A	
	Centola	2013	Wnt3a	FGF2, wnt3a	TGF-β1	Human	3D	In medium	Blocking canonical signaling results in loss of increased mesenchymal markers and proliferation	Increased	Neutral	Negative	Increased GAG/DNA ratio and proliferation, no change in SOX9 expression after replacing growth factors with TGF-β1. No induction without TGF-β1
				Wnt3a						Increased	Enhanced	Negative	
				FGF2	-		2D			Increased	N/A	N/A	
					-					Increased	N/A	N/A	
	Pei	2014	Wnt3a	-	TGF-β3	Human	3D	Expression measured	N/A	N/A	N/A	Positive	Expression wnt3a high on substrates with greatest chondrogenic potential
	Fischer	2002	Wnt3a	-	BMP2, wnt3a	Murine	3D	Overexpression	Canonical signaling indicated by lower GSK-3b activity	N/A	N/A	Positive	Increased PG production
				-	-					N/A	N/A	Neutral	
	Occhetta	2015	Wnt3a	FGF2	FGF2, wnt3a	Human	3D	In medium	Not confirmed	Increased	N/A	Neutral	Increased proliferation, but no effect on chondrogenesis after 3 days
	Qu	2013	Wnt3a	-	TGF-β3, wnt3a	Rat	3D	Overexpression	Inhibiting canonical signaling stops increased proliferation but not loss of chondrogenic phenotype, inhibition of Ca2+ is other way around	Increased	N/A	Negative	Increased proliferation, downregulation of chondrogenic markers and PG production
	Hwang	2005	Wnt3a	-	Wnt3a	Rat	3D	In medium	Inhibition of chondrogenesis is canonical independent, but dedifferentiation is canonical and c-Jun AP-1 (noncanonical JNK pathway) activity dependent	N/A	N/A	Negative	Decreased PG production
	Hsu	2013	Wnt3a	-	TGF-β3	Rat	3D	Expression measured	Canonical signaling active upon osteogenesis substrates while non-canonical more on chondrogenesis substrates	N/A	N/A	Negative	Wnt3a expression low in substrate with greatest chondrogenic potential

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Table 1. Continued

Cell type	Author	Year	Wnt	Addition during expansion	Addition during differentiation	Species	Culture	Supplementation method wnt3a	Signaling pathway	Proliferation	Cell potency	Chondrogenesis	Outcome summary
Embryonic stem cells	Waese	2011	Wnt3a	-	BMP4, wnt3a	Mouse	2D	In medium, wnt3a added from day 5	Not confirmed	N/A	Diminished	Positive	Increased expression of chondrogenic markers and PG production with wnt3a only when complemented with BMP4 addition
				-	Wnt3a			Wnt3a from day1		N/A		Negative	
	Tanaka	2009	Wnt3a	-	TGF-β3, wnt3a	Mouse	2D	In medium	Not confirmed	N/A	N/A	Positive	Wnt3a or BMP4 required for chondrogenesis
Chondrocytes	Nalesso	2011	Wnt3a	-	Wnt3a	Human		Overexpression	Canonical caused proliferation and non-canonical caused down regulation of chondrogenic markers	Increase	N/A	Negative	Increased proliferation, reduced expression chondrogenic markers and PG production
	Öztürk	2017	Wnt3a	-	Wnt3a	Unspecified	3D	In medium	Rho signaling (noncanonical PCP pathway) as inhibition enables regaining of chondrocyte phenotype	N/A	N/A	Negative	Loss of chondrogenic markers and decreased PG production
				-			2D			N/A	N/A	Negative	
	Hosseini-Farahabadi	2017	Wnt3a	-	Wnt3a	Chicken	3D	In medium	DKK1 is able to rescue wnt3a induced cartilage phenotype so canonical pathway involved	N/A	N/A	Negative	Reduced PG production
	Reinhold	2006	Wnt3a	-	Wnt3a	Mouse	2D	In medium	Canonical signaling represses chondrogenesis	N/A	N/A	Negative	Chondrogenic markers downregulated
	Snelling	2016	Wnt3a	-	Wnt3a	Human	3D	In medium	Canonical, as DKK3 rescues wnt3a induced loss of chondrogenic markers	N/A	N/A	Negative	PG production reduced
	Wang	2019	Wnt3a	-	Wnt3a	Mouse	3D	Overexpression	Reduced canonical signaling prevented loss of chondrogenic markers	N/A	N/A	Negative	Loss of chondrogenic markers
	Hwang	2005	Wnt3a	-	Wnt3a	Chicken	2D	In medium	Canonical and noncanonical JNK signaling	N/A	N/A	Negative	PG production decreased and suppression of chondrogenic markers
	Surman-Schmitt	2015	Wnt3a	-	Wnt3a	Chicken and mouse	3D	Overexpression	Blocking canonical signaling with Wif-1 impaired growth of mesenchymal precursor cells and neutralized inhibition of chondrogenesis	N/A	N/A	Negative	PG production decreased, and suppression of chondrogenic markers
				-	Wnt3a		2D			Increased	N/A	Negative	

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Table 1. Continued

Cell type	Author	Year	Wnt	Addition during expansion	Addition during differentiation	Species	Culture	Supplementation method wnt3a	Signaling pathway	Proliferation	Cell potency	Chondrogenesis	Outcome summary
	Miyamoto	2017	Wnt3a	-	Wnt3a	Human	2D	In medium	Repressing β-catenin inhibited wnt3a mediated loss of chondrogenic markers. Therefore, canonical signaling	N/A	N/A	Negative	PG production decreased and suppression of chondrogenic markers
	Takamatsu	2014	Wnt3a	-	Wnt3a	Human	2D	In medium	Inhibiting canonical signaling prevents cartilage degradation	N/A	N/A	Negative	PG production decreased
NP cells	Pizzute	2018	Wnt3a	-	Wnt3a	Human	3D	Overexpression	Not confirmed	Increase	N/A	Neutral	Enhanced proliferation, no difference in chondrogenic markers or PG production
	Hiyama	2011	Wnt3a	-	Wnt3a	Rat	2D	Overexpression	Activation of canonical signaling may be cause of cytostatic activity but not involved in PG synthesis associated with de- or regeneration	N/A	Neutral	Neutral	No difference in aggrecan expression
	Hiyama	2010	Wnt3a	-	Wnt3a	Rat	2D	Overexpression	ECM degradation markers increased upon canonical signaling and NP cell senescence however aggrecan was upregulated, no change in COL2A1	Decreased viability	N/A	Neutral	No change in chondrogenic markers, decreased viability
Mesenchymal stromal cells	Dickinson	2017	Wnt5a	FGF2	-	Human	3D	Cells separated based on expression of ROR2 receptors	ROR2 signaling	N/A	Increased	Positive	ROR2+ cells had an increased PG production
	Church	2002	Wnt5a	-	Wnt5a	Chicken	3D	Overexpression	Different non-canonical pathways	Neutral	N/A	Positive	Increased PG production and number of nodules, decreased expression hypertrophic markers
	Pei	2014	Wnt5a	-	TGF-β3	Human	3D	Expression measured	Not confirmed	N/A	N/A	Positive	Expression wnt5a high in substrates with greatest chondrogenic potential
	Hsu	2013	Wnt5a	-	TGF-β3	Rat	3D	Expression measured	Canonical signaling more activated on osteogenesic substrates while non-canonical more on chondrogenesic substrates	N/A	N/A	Positive	Expression wnt5a high in substrates with greatest chondrogenic potential

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Table 1. Continued

Cell type	Author	Year	Wnt	Addition during expansion	Addition during differentiation	Species	Culture	Supplementation method wnt3a	Signaling pathway	Proliferation	Cell potency	Chondrogenesis	Outcome summary
Embryonic stem cells (differentiated towards MSCs)	Gibson	2017	Wnt5a	bFGF	Wnt5a	Human	3D	In medium	Not confirmed	N/A	N/A	Onset positive later negative	Increased PG production, also increased chondrogenic markers, lost after longer culture periods
Chondrocytes	Bradley	2010	Wnt5a	-	Wnt5a	Mouse	3D	In medium	PKA pathway of non-canonical signaling with stage dependent effects. Stimulation in early stages and inhibition of hypertrophy at later stages	Neutral	N/A	Onset positive later negative	Increased PG production and chondrogenic markers which were lost after longer culture periods
			Wnt5a	-	Wnt5a		2D			Neutral	N/A		
	Hosseini-Farahabadi	2017	Wnt5a	-	Wnt5a	Chicken	3D	In medium	JNK/PCP pathway is suggested to be related with removal cartilage matrix, JNK is involved with ROR2 activation	Neutral	N/A	Negative	After 4 days no difference in PG production, at 6 days almost total loss PG production, cartilage matrix genes upregulated
NP cells	Pizzute	2018	Wnt5a	-	Wnt5a	Human	3D	Overexpression	Not confirmed	Decreased	N/A	Positive	Significantly less proliferation than control, enhanced chondrogenic and NP markers which decreased over time, and increased PG production
	Hiyama	2011	Wnt5a	-	Wnt5a	Rat	2D	Overexpression	Not confirmed	Neutral	N/A	Neutral	No difference in aggrecan expression
	Li	2018	Wnt5a	-	Wnt5a	Rat	2D	In medium	JNK signaling of the non-canonical PCP pathway, when inhibited, wnt5a upregulation is suppressed	N/A	N/A	Positive	Increased GAG production and upregulation of chondrogenic markers upon wnt5a addition

Papers are initially divided on the analyzed cell type and whether wnt3a or wnt5a is the subject. The table distinguishes reported effects on proliferation (i.e., increased, neutral, or decreased), cell potency (i.e., whether the cell potency is enhanced, neutral, or diminished), and chondrogenesis (i.e., a positive, neutral, negative, or initially positive and later negative effect).

FGF, fibroblast growth factor; TGF, transforming growth factor; 2D, 2-dimensonal; 3D, 3-dimensional; N/A, not available; PG, proteoglycan; GAG, glycosaminoglycan; GSK, glycogen synthase kinase; AP-1, activating protein-1; JNK, c-Jun N-terminal kinase; BMP, bone morphogenetic protein; DKK, dickkopf-related protein; Wif-1, Wnt inhibitory factor 1; ECM, extracellular matrix; ROR2, receptor tyrosine kinase-like orphan receptor 2; bFGF, basic fibroblast growth factor; PKA, protein kinase A; PCP, planar cell polarity; NP, nucleus pulposus.

the control.[16] However, *WNT5A* overexpression is beneficial for the redifferentiation of NP cells as shown by the upregulation of specific NP cell markers such as *PAX1* and *FOXF1* and matrix genes *COL2A1* and *ACAN*.[16] Moreover, the GAG/collagen II ratio, as a specific marker for healthy NP matrix (i.e., ~25:1 compared to a ratio of ~2:1 in juvenile cartilage[57]), is significantly increased after *WNT5A* overexpression[16] and lost upon lentiviral knockdown of *WNT5A*, as is the upregulation of the NP cell markers and matrix genes.[16]

CHONDROCYTES

In chondrocytes, the effects of wnt3a are described repeatedly to stimulate chondrogenic dedifferentiation.[13,27,45-49,58-60] The origin of the chondrocytes studied ranged from humans, large animals (i.e., bovine), to smaller animals (i.e., rat or chicken). Despite the differences in the supplementation method and the origin of the cells, all articles reported either a loss of chondrogenic gene marker expression, decreased proteoglycan production or a combination of these. However, addition of wnt3a appears to upregulate proliferation rate.[13,58] This could suggest that wnt3a inhibits the maturation state of chondrogenesis and instead gives rise to a more potent cell phenotype.

Wnt5a initially appears to be described with conflicting results in available literature, as wnt5a is shown to both promote and inhibit chondrocyte differentiation.[45,61] The discrepancy appears to originate from cellular differentiation stage at which wnt5a is introduced. Particularly, wnt5a appears to induce chondrogenic differentiation in early stages of differentiation whereas it inhibits maturation and inhibits hypertrophy in the end stages of chondrogenesis.[45,61] Contrariwise, blocking wnt5a decreased early chondrocyte differentiation but enhanced hypertrophy.[61] Noteworthy, the beneficial effects of wnt5a treatment are quite temporarily as enhanced chondrogenic markers expression was observed following wnt5a stimulation, which thereafter rapidly decreases.[61] Wnt5a is described to eventually stimulate enzymatic cartilage matrix degradation, inhibiting chondrogenesis.[45,62] These results demonstrate the stage-dependent influence of wnt5a in chondrocytes. *In vivo*, similar observations have been made.[12,45,63] Wnt5a is found to have a significant role in chondrogenesis where it follows a proximodistal gradient in the mesoderm of the developing limb.[64] Ectopically expression of *WNT5A* in chicken limb buds, results in malformation of the tissue i.e., reduced bone size and presenting a less matured phenotype.[12,63]

EMBRYONIC STEM CELLS

In ESCs, wnt3a is mainly reported to benefit expansion to generate a large number of chondrogenic potent cells. Moreover, wnt3a is stated to inhibit chondrogenesis, and instead induces cells to adopt a more immature phenotype.[26,65] Work by Tanaka et al.[65] demonstrated that wnt3a, especially in combination with the BMP4 inhibitor noggin (NOG), can generate lateral plate mesoderm phenotypes capable of differentiating towards a chondrogenic phenotype. Interestingly, Waese and Stanford[26] found that when first supplying the cells with BMP4 for 5 days and thereafter replacing BMP4 for wnt3a, chondrogenic potential could be optimized. Thus, suggesting that BMP4 is beneficial at the onset of differentiation while wnt3a acts as a late inducer of chondrogenesis, indicating that the crosstalk of BMP4 and wnt3a might be crucial for the chondrogenic differentiation of ESCs.

No work was identified that applied wnt5a directly onto ESC for chondrogenic differentiation. However, Gibson and colleagues[66] have applied wnt5a supplementation with pellet cultures of ESCs that were predifferentiated to take on a MSC phenotype. Similar effects were reported by wnt5a as observed with wnt3a[66] *viz.* promoting chondrogenesis and limiting hypertrophy. Chondrogenic markers *SOX9* and *COL2A1* increased whereas the expression of hypertrophy markers; *COL10A1* and *ALP*, decreased. In the same study,[66] the effect of BMP2 was found to upregulate early chondrogenic markers and proteoglycan production, attaining higher ratios than wnt5a. However, BMP2 also significantly increased expression of hypertrophic markers. The sequential treatment of BMP2 and wnt5a showed beneficial for inducing articular chondrocyte-like phenotypes, constituting initial chondrogenic induction via BMP2, followed by wnt5a supplementation on the fifth day to maintain the chondrogenic phenotype and preventing hypertrophy.[66]

MESENCHYMAL STEM CELLS

In MSCs, the chondrogenic effect of wnt3a is found to be the most variable across different studies (Table 1). The reporting agrees on the effects regarding the induction of proliferation. In all identified cases reporting on cell growth, wnt3a treatment lead to significantly higher proliferation rates.[21-23,67] Nevertheless, wnt3a does appear to have a negative effect on chondrogenesis according to two independent reports[21,49] applying either overexpression in rat MSCs[21] or supplementation in chicken embryo limb-bud MSCs.[49]

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Two studies[24,68] measuring *WNT3A* expression in MSCs after TGF-β3-mediated chondrogenesis on different substrates, showed opposing results as the samples with the highest chondrogenic potential were determined to have the lowest relative expression of *WNT3A* according to Hsu and Huang[68] or highest *WNT3A* expression in the paper of Pei et al.[24] in pellets with the most prominent chondrogenic characteristics. However, due to the differences in culture substrates and cell sourcing, these studies are not directly comparable.

Three papers[22,23,25] on wnt3a mediated induction of MSCs concluded an increase in chondrogenic potential caused by wnt3a when expanded in combination or subsequently replaced by a chondrogenic stimulating growth factor. Narcisi et al.[22] found that dual supplementation of wnt3a and FGF2 during expansion significantly enhanced the chondrogenic potential of MSCs. Contrarily, supplementation of wnt3a without FGF2, did not significantly stimulate GAG production nor *COL2* expression relative to non-induced MSCs. Similarly, MSC expansion with wnt3a followed by subsequent TGF-β1 stimulation similarly resulted in significant increased GAG deposition,[23] however, addition of FGF2 during wnt3a stimulated monolayer culture abolished these beneficial effects. In the research of Fischer et al.,[25] overexpression of *WNT3A* in combination with BMP2 in murine embryo derived MSCs, resulted in significantly higher proteoglycan production.[25] From these observations we hypothesize that wnt3a is not directly able to induce a chondrogenic phenotype, but rather has the capacity to enhance chondrogenic potential, stimulating chondrogenesis induced by other growth factors in human MSCs. As wnt3a appears to increase proliferation (Table 1) and reduce hypertrophic markers[22] it is possible that wnt3a maintains cell potency and thus enables MSCs to preserve a higher chondrogenic potential. When used during expansion and subsequently replaced with chondrogenic growth factor, wnt3a could reinforce chondrogenic potential which could be a strategy to optimize chondrogenic differentiation of MSCs.

Indications of the significance of wnt5a in chondrogenic MSC differentiation can be derived from Dickinson et al.[69] Here, ROR2 was identified as a cell surface marker on MSCs with an enhanced chondrogenic potential. After separation ROR2 positive MSCs were embedded in a collagen sponge and implanted in a prepared defect within the medial femoral condyle of sheep. After remaining *in vivo* for 3 months, the implants with increased ROR2 expression contained higher expression of chondrogenic markers.[69] Also, during TGF-β3 induced chondrogenic differentiation, wnt5a levels significantly increased, reaching higher levels than the control group of articular chondrocytes. A significant downregulation followed over time, while the expression in articular chondrocytes remained low,[70] further emphasizing the role of wnt5a in early chondrogenic differentiation. Finally, Hsu and Huang[68] examined MSCs seeded on different substrates and their ability to induce chondrogenic differentiation, and showed that samples presenting highest chondrogenic potency (chitosan grafted with higher densities of hyaluronan) presented a significantly higher expression of *WNT5A*.

Overall, the response of MSCs to wnt5a treatment appears relatively homogeneous. As discussed, wnt5a appears stimulatory towards chondrogenesis (specifically in early stages) while appearing to inhibit maturation and hypertrophy.[66] This is further emphasized by an *in vivo* study overexpressing *WNT5A* in chicken wing bud MSCs, resulting in a 1.5-fold enhanced alcian blue staining and increase in the number of nodules, whereas the activity levels of the maturation marker *ALP*, did not detectably change.[71] From this collection of data, wnt5a appears to play a crucial role in inducing chondrogenesis and is able to inhibit hypertrophic chondrogenesis.

Wnt3a AND Wnt5a AS TOOLS FOR REGENERATION OF THE INTERVERTEBRAL DISC

As mentioned previously, interest has been paid to the potential of growth factors[72,73] and cells[7,9] for regeneration of the IVD. Wnt3a and wnt5a are crucial factors for the maintenance and induction of cells to a chondrogenic phenotype and thus hold promise as tools in treatment of IVD degeneration. For the establishment of IVD regeneration via cell therapy, ideally NP cells are employed due to their intrinsic capacity to survive and thrive within the IVD.[74] Nevertheless, IVD cells are commonly derived from compromised tissue sources presenting low cellular yield and reduced cell potency.[10,74,75] Moreover, standard expansion of NP cells *in vitro* further reduces their overall potency.[75,76] From our current review, however, neither wnt3a nor wnt5a seem to have a clear beneficial effect on NP cell proliferation induction to enhance cell numbers, maintain overall potency, or induce a chondrogenic phenotype *in vitro* (Figs. 3A, 4A). Their effect *in vivo* remains largely undetermined. Li et al.[77] studied the effect of wnt5a on rat induced disc degeneration and found that administration of wnt5a could enhance the rate of aggrecan and type II collagen producing cells, as well as presenting enhanced magnetic resonance imaging index maintenance. Moreover, the authors nicely presented the ability of

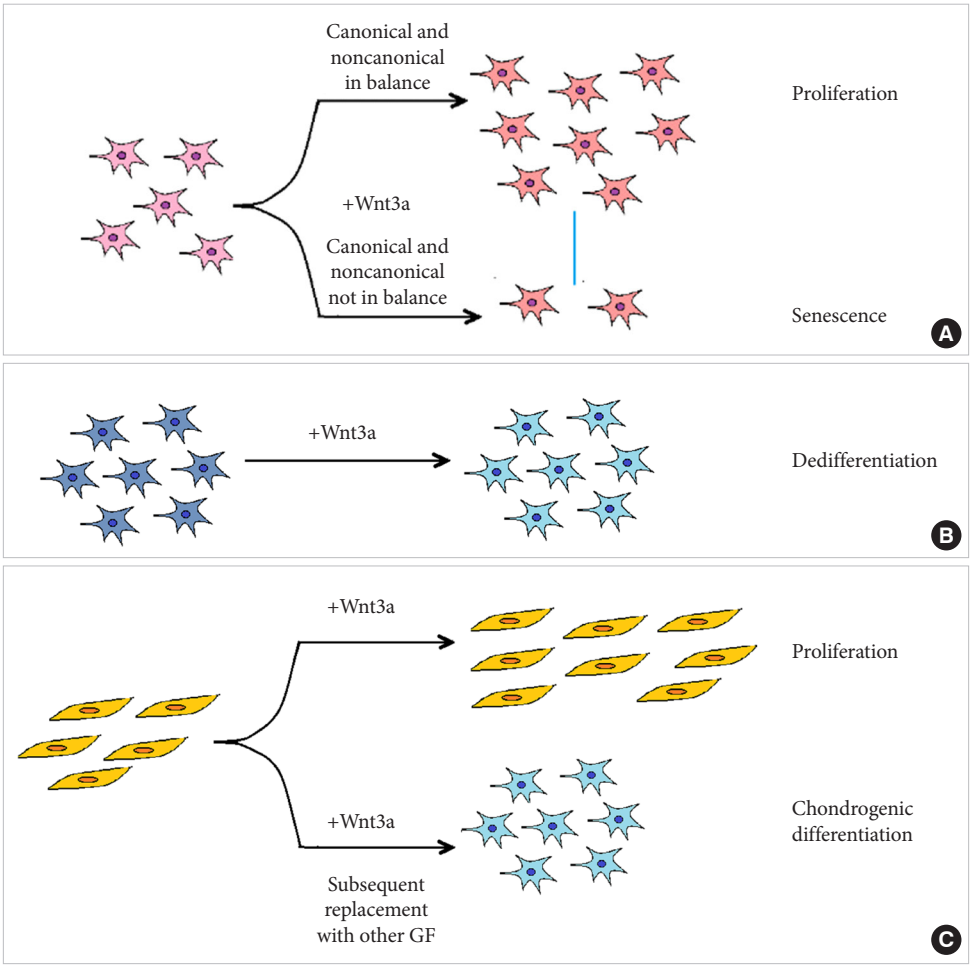

Fig. 3. A simplified summary of wnt3a effects on different cell types under specific conditions towards potential regeneration of the intervertebral disc, concluded from available literature. (A) Nucleus pulposus cells are reported to not change in their chondrogenic potential, but can start proliferation upon wnt3a stimulation. However, when only one pathway is stimulated in particular, cell senescence can occur. (B) Chondrocytes are determined to start dedifferentiating after wnt3a supplementation. The cells become more potent but also less chondrogenic. (C) Mesenchymal stromal cells are observed to have different reactions upon wnt3a treatment. There appears to be a pattern as proliferation is upregulated after wnt3a supplementation, while chondrogenic differentiation occurs when wnt3a is supplemented and subsequently replaced with other growth factors (GFs).

wnt5a to limit IVD inflammation by inhibiting the action of TNFα. Nevertheless, *in vivo* application does require caution, as multiple reports have presented the ability of wnt5a to induce or support osteogenesis,[78-80] tissue-fibrosus,[81,82] and M2 polarization of macrophages.[83] Similarly, wnt3a has been associated with the progression of a variety of tumors.[84]

On the contrary, wnt3a and wnt5a influence on chondrocytes presents a more apparent effect. Chondrocytes resemble NP cells, as both reside in avascular tissues of which the function is to distribute and transfer biomechanical pressure,[85] appointing chondrocytes as a potential alternative to NP cells. Opposingly, chondrocytes do present a lower ratio of PG vs collagen production[57] and are endemically developed to adapt to distinctly different biomechanical forces as suggested by the matrix organization in articular cartilage. Moreover, altering these behavior facets of mature cells might be particularly challenging. From our review the applications of wnts as a method to enhance chondrocytes regenerative capacity is not suggested to be a viable option. Wnt3a, although able to enhance cell proliferation, evidently has a negative impact on the chondrogenic features of the cells (Fig. 3B). Although, wnt5a does not seem to impact cell proliferation, it does reduce its chondrogenic phenotype (Fig. 4B). As such, preconditioning with either of these ligands appears delicate and stimulating chondrocytes with either wnt3a or wnt5a is likely not to be efficient to enhance the capacity of chondrocytes for cellular products towards IVD repair.

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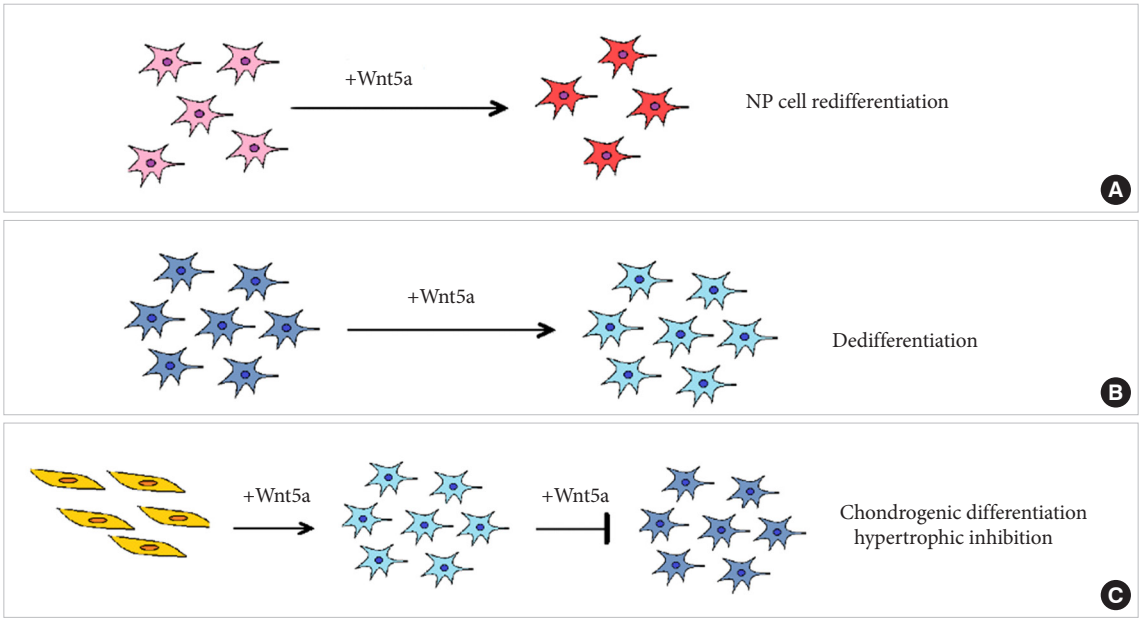

Fig. 4. A simplified summary of wnt5a effects on different cell types under specific conditions towards potential regeneration of the intervertebral disc, concluded from available literature. (A) Wnt5a induces redifferentiation of nucleus pulposus (NP) cells, but proliferation is reported to decrease compared to the control. (B) Chondrocytes are determined to start dedifferentiating after wnt5a supplementation. The cells become more potent but also less chondrogenic. (C) In mesenchymal stromal cells, wnt5a is solely capable of inducing chondrogenic differentiation in early stages and inhibits maturation and hypertrophy, maintaining a more potent chondrogenic state.

However, wnt3a and wnt5a under appropriate conditions appear to hold promise for the induction of MSC differentiation to adopt a more chondrogenic phenotype. MSCs have distinct advantages to differentiated NP cells or chondrocytes, such as easy accessibility, a high cell potency,[86] and immunomodulatory properties.[87] Moreover, MSCs possess multipotent differentiation capabilities, including towards NP cell-like phenotypes,[88-90] although, the precise nature of these chondrogenic MSCs remains ill-defined. Moreover, it remains undetermined whether the primary effect of transplanted cells is their immunomodulatory effects, endemic cell reactivation, active contribution to matrix production, or a combination thereof.[91-93] Nonetheless, MSCs are being explored in clinical setting, and their primary data suggest MSC transplantation to be safe and able to provoke clinical improvements.[7] In order to enhance the potency of MSCs to succeed in the IVD, multiple studies have explored the potential of preconditioning cells prior to IVD transplantation.[94] Our review suggests that sole supplementation of wnt3a to MSCs consistently enhanced their proliferative capacity. However, wnt3a presented a relative negative effect on chondrogenic characteristics. Interestingly, however, is the capacity of wnt3a to maintain (chondrogenic) potency of MSCs in culture, which potentially enables enhanced chondrogenic differentiation upon the subsequent or combined supplementation of an additional (chondrogenic) growth factor (Fig. 3C). On the other hand, the positive relation of wnt5a on chondrogenesis is homogeneously supported by our identified papers.[19,20] Moreover, data suggest wnt5a interferes with hypertrophic maturation (Fig. 4C).[71] Although, verification is required on the ability of wnt5a and wnt3a to optimize MSC-based cell therapy products for the IVD regeneration, current *in vitro* results do suggest beneficial effects with regard to maintaining MSC potency by wnt3a or stimulating chondrogenic features by wnt5a.

CONCLUDING REMARKS

In this review, we present the role of wnt3a and wnt5a as potential chondrogenic stimulators and for the potential use as NP cell and MSC inducers. Wnt3a and wnt5a are each found able to support NP cell induction in their respective ways, however, their effect appears highly context dependent and varies dependent cell type. Data suggest that the application of wnt3a primarily has the ability to enhance proliferation and maintain potency. Although, undesirable loss in chondrogenesis is observed in chondrogenic cells, including NP cells, wnt3a appears to be able to prolong MSC chondrogenic potency with extend-

ed culture. On the contrary, wnt5a appears to restrict NP cell and chondrocytes chondrogenic features, while for MSCs it induces early chondrogenic stimulation. Although preconditioning of MSCs is actively being explored[88,95,96] and preculturing of MSCs under hypoxic conditions proved to be safe and promising in human clinical trials against IVD degeneration,[97] more research is required to confirm the beneficial effects of wnt3a and wnt5a as NP cell inducers, and the competence of these stimulated cells to thrive in a degenerative IVD. Elucidation of the regulatory pathways involved in wnt-responses could provide new insights and targets for tackling IVD degeneration and producing regenerative strategies for IVD repair. Once a thorough understanding of the effect of wnt3a and wnt5a treated cells *in vivo* is achieved, the use of these growth factors may serve as a valuable tool as chondrogenic stimulators for NP cell induction.

CONFLICT OF INTEREST

The authors have nothing to disclose.

REFERENCES

1. Reisbord LS, Greenland S. Factors associated with self-reported back-pain prevalence: a population-based study. J Chronic Dis 1985;38:691-702.
2. Tong W, Lu Z, Qin L, et al. Cell therapy for the degenerating intervertebral disc. Transl Res 2017;181:49-58.
3. Balagué F, Mannion AF, Pellisé F, et al. Clinical update: low back pain. Lancet (London, England) 2007;369:726-8.
4. Choi H, Johnson ZI, Risbud MV. Understanding nucleus pulposus cell phenotype: a prerequisite for stem cell based therapies to treat intervertebral disc degeneration. Curr Stem Cell Res Ther 2015;10:307-16.
5. Dehghani F, Fathi A. Challenges for cartilage regeneration. In: Li Q., Mai YW, editors. Biomaterials for implants and scaffolds. Springer Series in Biomaterials Science and Engineering, vol 8. Heidelberg: Springer; 2017. p.389-466.
6. Dowdell J, Erwin M, Choma T, et al. Intervertebral disk degeneration and repair. Neurosurgery 2017;80:S46-54.
7. Schol J, Sakai D. Cell therapy for intervertebral disc herniation and degenerative disc disease: clinical trials. Int Orthop 2019;43:1011-25.
8. Thorpe AA, Bach FC, Tryfonidou MA, et al. Leaping the hurdles in developing regenerative treatments for the intervertebral disc from preclinical to clinical. JOR spine 2018;1:e1027.
9. Smith LJ, Silverman L, Sakai D, et al. Advancing cell therapies for intervertebral disc regeneration from the lab to the clinic: recommendations of the ORS spine section. JOR spine 2018;1:e1036.
10. Hiraishi S, Schol J, Sakai D, et al. Discogenic cell transplantation directly from a cryopreserved state in an induced intervertebral disc degeneration canine model. JOR spine 2018;1:e1013.
11. Rudnicki JA, Brown AMC. Inhibition of chondrogenesis by Wnt gene expression in vivo and in vitro. Dev Biol 1997;185:104-18.
12. Hartmann C, Tabin CJ. Dual roles of Wnt signaling during chondrogenesis in the chicken limb. Development 2000;127:3141-59.
13. Nalesso G, Sherwood J, Bertrand J, et al. WNT-3A modulates articular chondrocyte phenotype by activating both canonical and noncanonical pathways. J Cell Biol 2011;193:551-64.
14. Wang Y, Fan X, Xing L, et al. Wnt signaling: a promising target for osteoarthritis therapy. Cell Commun Signal 2019;17:97.
15. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781-810.
16. Pizzute T, He F, Zhang X-B, et al. Impact of Wnt signals on human intervertebral disc cell regeneration. J Orthop Res 2018;36:3196-207.
17. Hartmann C, Tabin CJ. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. Cell 2001;104:341-51.
18. Stott NS, Jiang TX, Chuong CM. Successive formative stages of precartilaginous mesenchymal condensations in vitro: modulation of cell adhesion by Wnt-7A and BMP-2. J Cell Physiol 1999;180:314-24.
19. Staines KA, Macrae VE, Farquharson C. Cartilage development and degeneration: a Wnt Wnt situation. Cell Biochem Funct 2012;30:633-42.
20. Green JD, Tollemar V, Dougherty M, et al. Multifaceted signaling regulators of chondrogenesis: implications in cartilage regeneration and tissue engineering. Genes Dis 2015;2:307-27.
21. Qu F, Wang J, Xu N, et al. WNT3A modulates chondrogenesis via canonical and non-canonical Wnt pathways in MSCs. Front Biosci (Landmark Ed) 2013;18:493-503.
22. Narcisi R, Cleary MA, Brama PAJ, et al. Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. Stem cell reports 2015;4:459-72.

 https://doi.org/10.14245/ns.2040040.020

23. Centola M, Tonnarelli B, Schären S, et al. Priming 3D cultures of human mesenchymal stromal cells toward cartilage formation via developmental pathways. Stem Cells Dev 2013; 22:2849-58.
24. Pei M, Li J, Zhang Y, et al. Expansion on a matrix deposited by nonchondrogenic urine stem cells strengthens the chondrogenic capacity of repeated-passage bone marrow stromal cells. Cell Tissue Res 2014;356:391-403.
25. Fischer L, Boland G, Tuan RS. Wnt-3A enhances bone morphogenetic protein-2-mediated chondrogenesis of murine C3H10T1/2 mesenchymal cells. J Biol Chem 2002;277:30870-8.
26. Waese EYL, Stanford WL. One-step generation of murine embryonic stem cell-derived mesoderm progenitors and chondrocytes in a serum-free monolayer differentiation system. Stem Cell Res 2011;6:34-49.
27. Öztürk E, Despot-Slade E, Pichler M, et al. RhoA activation and nuclearization marks loss of chondrocyte phenotype in crosstalk with Wnt pathway. Exp Cell Res 2017;360:113-24.
28. Kondo N, Yuasa T, Shimono K, et al. Intervertebral disc development is regulated by Wnt/β-catenin signaling. Spine (Phila Pa 1976) 2011;36:E513-8.
29. Dahia CL, Mahoney EJ, Durrani AA, et al. Intercellular signaling pathways active during intervertebral disc growth, differentiation, and aging. Spine (Phila Pa 1976) 2009;34: 456-62.
30. Holguin N, Silva MJ. In-vivo nucleus pulposus-specific regulation of adult murine intervertebral disc degeneration via Wnt/beta-catenin signaling. Sci Rep 2018;8:11191.
31. Hiyama A, Sakai D, Tanaka M, et al. The relationship between the Wnt/β-catenin and TGF-β/BMP signals in the intervertebral disc cell. J Cell Physiol 2011;226:1139-48.
32. Hiyama A, Sakai D, Risbud MV, et al. Enhancement of intervertebral disc cell senescence by WNT/β-catenin signaling-induced matrix metalloproteinase expression. Arthritis Rheum 2010;62:3036-47.
33. Niehrs C. The complex world of WNT receptor signalling. Nat Rev Mol Cell Biol 2012;13:767-79.
34. Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006;127:469-80.
35. Aberle H, Bauer A, Stappert J, et al. beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J 1997;16: 3797-804.
36. He X. LDL receptor-related proteins 5 and 6 in Wnt/ -catenin signaling: arrows point the way. Development 2004;131:1663-77.
37. Kohn AD, Moon RT. Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium 2005;38:439-46.
38. Moon RT, Kohn AD, De Ferrari GV, et al. WNT and β-catenin signalling: diseases and therapies. Nat Rev Genet 2004;5:691-701.
39. Komiya Y, Habas R. Wnt signal transduction pathways. Organogenesis 2008;4:68-75.
40. Chen AE, Ginty DD, Fan CM. Protein kinase A signalling via CREB controls myogenesis induced by Wnt proteins. Nature 2005;433:317-22.
41. Weivoda MM, Ruan M, Hachfeld CM, et al. Wnt signaling inhibits osteoclast differentiation by activating canonical and noncanonical cAMP/PKA pathways. J Bone Miner Res 2016;31:65-75.
42. Stricker S, Rauschenberger V, Schambony A. ROR-family receptor tyrosine kinases. Curr Top Dev Biol 2017;123:105-42.
43. Ford CE, Qian Ma SS, Quadir A, et al. The dual role of the novel Wnt receptor tyrosine kinase, ROR2, in human carcinogenesis. Int J cancer 2013;133:779-87.
44. Kim JG, Kim MJ, Choi W, et al. Wnt3A induces GSK-3β phosphorylation and β-catenin accumulation through RhoA/ROCK. J Cell Physiol 2017;232:1104-13.
45. Hosseini-Farahabadi S, Geetha-Loganathan P, Fu K, et al. Dual functions for WNT5A during cartilage development and in disease. Matrix Biol 2013;32:252-64.
46. Reinhold MI, Kapadia RM, Liao Z, et al. The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis. J Biol Chem 2006;281:1381-8.
47. Snelling SJB, Davidson RK, Swingler TE, et al. Dickkopf-3 is upregulated in osteoarthritis and has a chondroprotective role. Osteoarthritis Cartilage 2016;24:883-91.
48. Wang X, Cornelis FMF, Lories RJ, et al. Exostosin-1 enhances canonical Wnt signaling activity during chondrogenic differentiation. Osteoarthritis Cartilage 2019;27:1702-10.
49. Hwang SG, Yu SS, Lee SW, et al. Wnt-3a regulates chondrocyte differentiation via c-Jun/AP-1 pathway. FEBS Lett 2005; 579:4837-842.
50. Hiyama A, Yokoyama K, Nukaga T, et al. A complex interaction between Wnt signaling and TNF-α in nucleus pulposus cells. Arthritis Res Ther 2013;15:R189.
51. Evashwick-Rogler TW, Lai A, Watanabe H, et al. Inhibiting tumor necrosis factor-alpha at time of induced intervertebral disc injury limits long-term pain and degeneration in a rat model. JOR spine 2018;1:e1014.
52. Arai F, Hiyama A, Sakai D, et al. The expression and role of non-canonical (PKC) signaling in nucleus pulposus cell metabolism. J Orthop Res 2012;30:1478-85.

53. Houschyar KS, Tapking C, Borrelli MR, et al. Wnt Pathway in bone repair and regeneration - what do we know so far. Front cell Dev Biol 2018;6:170.
54. Sun Z, Jian Y, Fu H, et al. MiR-532 downregulation of the Wnt/β-catenin signaling via targeting Bcl-9 and induced human intervertebral disc nucleus pulposus cells apoptosis. J Pharmacol Sci 2018;138:263-70.
55. Zhan S, Wang K, Song Y, et al. Long non-coding RNA HOTAIR modulates intervertebral disc degenerative changes via Wnt/β-catenin pathway. Arthritis Res Ther 2019;21:201.
56. DiRenzo DM, Chaudhary MA, Shi X, et al. A crosstalk between TGF-β/Smad3 and Wnt/β-catenin pathways promotes vascular smooth muscle cell proliferation. Cell Signal 2016; 28:498-505.
57. Risbud MV, Schoepflin ZR, Mwale F, et al. Defining the phenotype of young healthy nucleus pulposus cells: recommendations of the Spine Research Interest Group at the 2014 annual ORS meeting. J Orthop Res 2015;33:283-93.
58. Surmann-Schmitt C, Widmann N, Dietz U, et al. Wif-1 is expressed at cartilage-mesenchyme interfaces and impedes Wnt3a-mediated inhibition of chondrogenesis. J Cell Sci 2009;122(Pt 20):3627-37.
59. Miyamoto K, Ohkawara B, Ito M, et al. Fluoxetine ameliorates cartilage degradation in osteoarthritis by inhibiting Wnt/β-catenin signaling. PLoS One 2017;12:e0184388.
60. Takamatsu A, Ohkawara B, Ito M, et al. Verapamil protects against cartilage degradation in osteoarthritis by inhibiting Wnt/β-catenin signaling. PLoS One 2014;9:e92699.
61. Bradley EW, Drissi MH. WNT5A regulates chondrocyte differentiation through differential use of the CaN/NFAT and IKK/NF-kappaB pathways. Mol Endocrinol 2010;24:1581-93.
62. Mao G, Zhang Z, Hu S, et al. Exosomes derived from miR-92a-3p-overexpressing human mesenchymal stem cells enhance chondrogenesis and suppress cartilage degradation via targeting WNT5A. Stem Cell Res Ther 2018;9:247.
63. Kawakami Y, Wada N, Nishimatsu SI, et al. Involvement of Wnt-5a in chondrogenic pattern formation in the chick limb bud. Dev Growth Differ 1999;41:29-40.
64. Al-Qattan MM. WNT pathways and upper limb anomalies. J Hand Surg Eur Vol 2011;36:9-22.
65. Tanaka M, Jokubaitis V, Wood C, et al. BMP inhibition stimulates WNT-dependent generation of chondrogenic mesoderm from embryonic stem cells. Stem Cell Res 2009;3:126-41.
66. Gibson JD, O'Sullivan MB, Alaee F, et al. Regeneration of articular cartilage by human ESC-derived mesenchymal progenitors treated sequentially with BMP-2 and Wnt5a. Stem Cells Transl Med 2017;6:40-50.
67. Occhetta P, Centola M, Tonnarelli B, et al. High-throughput microfluidic platform for 3D cultures of mesenchymal stem cells, towards engineering developmental processes. Sci Rep 2015;5:10288.
68. Hsu S, Huang GS. Substrate-dependent Wnt signaling in MSC differentiation within biomaterial-derived 3D spheroids. Biomaterials 2013;34:4725-38.
69. Dickinson SC, Sutton CA, Brady K, et al. The Wnt5a receptor, receptor tyrosine kinase-like orphan receptor 2, is a predictive cell surface marker of human mesenchymal stem cells with an enhanced capacity for chondrogenic differentiation. Stem Cells 2017;35:2280-91.
70. Diederichs S, Tonnier V, März M, et al. Regulation of WNT5A and WNT11 during MSC in vitro chondrogenesis: WNT inhibition lowers BMP and hedgehog activity, and reduces hypertrophy. Cell Mol Life Sci 2019;76:3875-89.
71. Church V, Nohno T, Linker C, et al. Wnt regulation of chondrocyte differentiation. J Cell Sci 2002;115(Pt 24):4809-18.
72. Kennon JC, Awad ME, Chutkan N, et al. Current insights on use of growth factors as therapy for intervertebral disc degeneration. Biomol Concepts 2018;9:43-52.
73. Hodgkinson T, Shen B, Diwan A, et al. Therapeutic potential of growth differentiation factors in the treatment of degenerative disc diseases. JOR spine 2019;2:e1045.
74. Sakai D, Schol J. Cell therapy for intervertebral disc repair: clinical perspective. J Orthop Transl 2017;9:8-18.
75. Sakai D, Schol J, Bach FC, et al. Successful fishing for nucleus pulposus progenitor cells of the intervertebral disc across species. JOR spine 2018;1:e1018.
76. Nukaga T, Sakai D, Schol J, et al. Minimal sustainability of dedifferentiation by ROCK inhibitor on rat nucleus pulposus cells in vitro. Spine Surg Relat Res. 2019;3:385-91.
77. Li Z, Zhang K, Li X, et al. Wnt5a suppresses inflammation-driven intervertebral disc degeneration via a TNF-α/NF-κB-Wnt5a negative-feedback loop. Osteoarthritis Cartilage 2018; 26:966-77.
78. Keller KC, Ding H, Tieu R, et al. Wnt5a supports osteogenic lineage decisions in embryonic stem cells. Stem Cells Dev 2016;25:1020-32.
79. Bolzoni M, Donofrio G, Storti P, et al. Myeloma cells inhibit non-canonical wnt co-receptor ror2 expression in human bone marrow osteoprogenitor cells: effect of wnt5a/ror2 pathway activation on the osteogenic differentiation impairment

 https://doi.org/10.14245/ns.2040040.020

induced by myeloma cells. Leukemia 2013;27:451-63.
80. Nemoto E, Ebe Y, Kanaya S, et al. Wnt5a signaling is a substantial constituent in bone morphogenetic protein-2-mediated osteoblastogenesis. Biochem Biophys Res Commun 2012;422:627-32.
81. Guan S, Zhou J. Frizzled-7 mediates TGF-β-induced pulmonary fibrosis by transmitting non-canonical Wnt signaling. Exp Cell Res 2017;359:226-34.
82. Rydell-Törmänen K, Zhou X-H, Hallgren O, et al. Aberrant nonfibrotic parenchyma in idiopathic pulmonary fibrosis is correlated with decreased β-catenin inhibition and increased Wnt5a/b interaction. Physiol Rep 2016;4:1-9.
83. Feng Y, Liang Y, Zhu X, et al. The signaling protein Wnt5a promotes TGFβ1-mediated macrophage polarization and kidney fibrosis by inducing the transcriptional regulators Yap/Taz. J Biol Chem 2018;293:19290-302.
84. Qi L, Sun B, Liu Z, et al. Wnt3a expression is associated with epithelial-mesenchymal transition and promotes colon cancer progression. J Exp Clin Cancer Res 2014;33:107.
85. Chen S, Fu P, Wu H, et al. Meniscus, articular cartilage and nucleus pulposus: a comparative review of cartilage-like tissues in anatomy, development and function. Cell Tissue Res 2017;370:53-70.
86. Lavrentieva A, Hatlapatka T, Neumann A, et al. Potential for osteogenic and chondrogenic differentiation of MSC. Adv Biochem Eng Biotechnol 2013;129:73-88.
87. Li N, Hua J. Interactions between mesenchymal stem cells and the immune system. Cell Mol Life Sci 2017;74:2345-60.
88. Colombier P, Clouet J, Boyer C, et al. TGF-β1 and GDF5 act synergistically to drive the differentiation of human adipose stromal cells toward nucleus pulposus-like cells. Stem Cells 2016;34:653-67.
89. Richardson SM, Hughes N, Hunt JA, et al. Human mesenchymal stem cell differentiation to NP-like cells in chitosan-glycerophosphate hydrogels. Biomaterials 2008;29:85-93.
90. Korecki CL, Taboas JM, Tuan RS, et al. Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype. Stem Cell Res Ther 2010;1:18.
91. Shu CC, Dart A, Bell R, et al. Efficacy of administered mesenchymal stem cells in the initiation and co-ordination of repair processes by resident disc cells in an ovine (Ovis aries) large destabilizing lesion model of experimental disc degeneration. JOR spine 2018;1:e1037.
92. Vickers L, Thorpe AA, Snuggs J, et al. Mesenchymal stem cell therapies for intervertebral disc degeneration: consideration of the degenerate niche. JOR spine 2019;2:e1055.
93. Loibl M, Wuertz-Kozak K, Vadala G, et al. Controversies in regenerative medicine: should intervertebral disc degeneration be treated with mesenchymal stem cells? JOR spine 2019;2:e1043.
94. Mochida J, Sakai D, Nakamura Y, et al. Intervertebral disc repair with activated nucleus pulposus cell transplantation: a three-year, prospective clinical study of its safety. Eur Cell Mater 2015;29:202-12; discussion 212.
95. Clarke LE, McConnell JC, Sherratt MJ, et al. Growth differentiation factor 6 and transforming growth factor-beta differentially mediate mesenchymal stem cell differentiation, composition, and micromechanical properties of nucleus pulposus constructs. Arthritis Res Ther 2014;16:R67.
96. Stoyanov J V, Gantenbein-Ritter B, Bertolo A, et al. Role of hypoxia and growth and differentiation factor-5 on differentiation of human mesenchymal stem cells towards intervertebral nucleus pulposus-like cells. Eur Cell Mater 2011; 21:533-47.
97. Elabd C, Centeno CJ, Schultz JR, et al. Intra-discal injection of autologous, hypoxic cultured bone marrow-derived mesenchymal stem cells in five patients with chronic lower back pain: a long-term safety and feasibility study. J Transl Med 2016;14:253.