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 pulposus (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-ages¹ 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
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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. Nevertheless, an optimal cell type or preconditioning method to enable optimized regeneration of NP tissue has not yet been established.

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. Both the activation and inhibition of wnt signaling result in cartilage breakdown. 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. The diverse wnt family, consisting of 19 members in homo sapiens, 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. On the contrary, Wnt7a and wnt14 are reported to inhibit or reverse chondrogenesis. Wnt3a, however, is particularly of interest due to numerous contradictions in the literature. Most studies tend to agree upon the positive regulatory effect of wnt3a on cell proliferation, however, both wnt3a activation and inhibition have been reported to downregulate cartilage phenotypic markers, and reduce glycosaminoglycan (GAG) production. Interestingly, in combination with other growth factors; e.g., fibroblast growth factor (FGF), transforming growth factor (TGF)-β, bone morphogenetic protein (BMP), Wnt3a appears to be beneficial for MSCs to adopt a chondrogenic phenotype in 2-dimensional, and 3-dimensional cultures. 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 are pivotal for cellular development, function, and tissue homeostasis, in various tissues, including cartilage tissues such as the IVD. Specifically during early development, IVD cells are highly dependent on wnt signaling for appropriate development. During these early stages, high activity of wnt is interfering with bone and cartilage development. The diverse wnt family, consisting of 19 members in homo sapiens, acts regulatory mechanisms involved in wnt-mediated 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. 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. 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. 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).

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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. 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. Interaction with Frizzled can lead to the activation of calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC), referred to as the wnt/calcium pathway. Noteworthy is that this pathway is found to inhibit canonical signaling. 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. These processes are suggested to play a crucial role in cytoskeletal organization and cell adhesion as these are linked to actin polymerization.
protein kinase A (PKA) pathway. Frizzled activates adenylate cyclase, which triggers cyclic adenosine monophosphate/PKA inhibiting the transcription factor NFATc1. Aside from Frizzled, wnts are shown to activate receptor tyrosine kinase-like orphan receptor 2 (ROR2), with wnt5a as their primary ligand. 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, 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. However, wnt5a and wnt5b have been reported to induce chondrogenesis in developmental stages, whereas it inhibits chondrogenesis in later stages. 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
reviews reporting contradictory conclusions, stating that wnt3a stimulates, but also inhibits chondrogenic differentiation. Another unusual characteristic of wnt3a is the capacity to activate both the canonical and noncanonical pathway, 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. Similarly by applying a GSK-3 inhibitor could alleviate wnt3a repression of chondrogenesis, and was found to effectuate its regulation via β-catenin pathway. Furthermore, decreased proteoglycan production caused by wnt3a could be recovered after addition of DKK3. Recently, a knockdown of exotosin-1 (Ext1), encoding a glycosyltransferase required for heparin sulfate (HS) chain elongation in HS-proteoglycan biosynthesis, was determined to downregulate canonical wnt signaling activation but upregulate markers for chondrogenesis. 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. Expression of COL2A1, aggrecan, and SOX9 all improved after Ca2+/CaMKII inhibition at higher rates than DKK1-mediated inhibition of canonical signaling. Besides the CaMKII pathway, the PCP pathway has also been demonstrated to mediate differentiation in chondrocytes. JNK inhibition resulted in the rescue of wnt3a downregulated chondrogenic markers SOX9 and COL2A1. 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, 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 or wnt3a supplementation via medium are not affected in chondrogenic marker expression or 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. Solely upregulation of wnt/β-catenin signaling caused no proliferation in rat-derived NP cells however, cell viability was determined to decrease with 50%. Moreover, wnt/β-catenin signaling was demonstrated to regulate tumor necrosis factor (TNF)-α, linking it to IVD degeneration, 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. However, this phenomenon has not been studied in human NP cells. Studies conducted on human cells primarily focus on the canonical pathway, in which contradictory results are obtained as specific downregulation of wnt/β-catenin signaling leads to NP cell apoptosis, while activation of wnt/β-catenin signaling is also reported to result in NP cell apoptosis. Canonical and noncanonical signaling appears to have cross-talk 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 signaling whereas overexpression of R-Smad, an intracellular signaling protein downstream of the BMP/TGF signaling pathway, inhibits wnt/β-catenin signaling. 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. Overexpression of WNT5A in human NP cells results, in contrast to WNT3A, in the reduction of proliferation compared to
| 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                                                                         |
| 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      | N/A            | Increase GAG/DNA ratio and proliferation, no change in SOX9 expression after replacing growth factors with TGF-β1 |                                                                 |
|                                   |         |      | FGF2                          | -                               | Human   | 2D      |                                |                                                                                   | Increased     | Neutral      | Negative       |                                                                                                                                            |
| Pei                               | 2014    | Wnt3a | TGF-β3                         | Human                           | 3D      | Expression measured           | N/A                                          | Increased     | N/A          | N/A            | Expression wnt3a high on substrates with greatest chondrogenic potential                                                                 |
| Fischer                           | 2002    | Wnt3a | -                             | BMP2, wnt3a                     | Murine  | 3D      | Overexpression                 | Canonical signaling indicated by lower GSK-3β activity                              | N/A           | N/A          | Positive       | Increased PG production                                                                                                              |
| 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 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                                                                   |

(Continued to the next page)
| Cell type               | Author                  | Year | Wnt Addition during expansion | Species | Culture | Supplementation method or wnt3a | Signaling pathway | Proliferation | Cell potency | Chondrogenesis | Outcome summary                                                                 |
|------------------------|-------------------------|------|-------------------------------|---------|---------|---------------------------------|-------------------|---------------|--------------|---------------|---------------------------------------------------------------------------------------------------------------------------------------|
| Embryonic stem cells   | Waese                   | 2011 | 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                       |
|                        | Tanaka                  | 2009 | Wnt3a                         | Mouse   | 2D      | Wnt3a from day1                  | N/A               | N/A           | Negative     | Positive      | Wnt3a or BMP4 required for chondrogenesis                                                                                               |
| Chondrocytes           | Nalesso                 | 2011 | Wnt3a                         | Human   | Overexpression | Canonical caused proliferation and non-canonical caused down regulation of chondrogenic markers | Increase | N/A           | Negative     | Positive      | Increased proliferation, reduced expression chondrogenic markers and PG production                                                                 |
|                        | Öztürk                  | 2017 | Wnt3a                         | Unspecified | 3D      | Rho signaling (noncanonical PCP pathway) as inhibition enables regaining of chondrocyte phenotype | N/A               | N/A           | Negative     | Negative      | Loss of chondrogenic markers and decreased PG production                                                                             |
|                        | Hosseini-Farahabadi     | 2017 | Wnt3a                         | Chicken | 3D      | In medium                         | N/A               | N/A           | Negative     | Reduced PG production                                                                                                                  |
|                        | Reinhold                | 2006 | Wnt3a                         | Mouse   | 2D      | In medium                         | Canonical signaling represses chondrogenesis | N/A           | Negative     | Chondrogenic markers downregulated                                                                                                      |
|                        | Snelling                | 2016 | Wnt3a                         | Human   | 3D      | In medium                         | Canonical, as DKK3 rescues wnt3a induced loss of chondrogenic markers | N/A           | Negative     | PG production reduced                                                                                                                  |
|                        | Wang                    | 2019 | Wnt3a                         | Mouse   | 3D      | Overexpression                    | Reduced canonical signaling prevented loss of chondrogenic markers | N/A           | Negative     | Loss of chondrogenic markers                                                                                                          |
|                        | Hwang                   | 2005 | Wnt3a                         | Chicken | 2D      | In medium                         | Canonical and noncanonical JNK signaling | N/A           | Negative     | PG production decreased and suppression of chondrogenic markers                                                                       |
|                        | Surman-Schmitt          | 2015 | 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       | Negative     | PG production decreased, and suppression of chondrogenic markers                                                                     |

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| Cell type                        | Author         | Year | Wnt      | Species | Culture | Addition during expansion | Addition during differentiatation | Supplementation method wnt3a | Signaling pathway                                                                 | Proliferation | Cell potency | Chondrogenesis | Outcome summary                                                                 |
|---------------------------------|----------------|------|----------|---------|---------|---------------------------|----------------------------------|-------------------------------|---------------------------------------------------------------------------------|---------------|--------------|----------------|-----------------------------------------------------------------------------|
|                                 | Miya-moto      | 2017 | Wnt3a    | Human   | 2D      | -                         | Wnt3a                           | 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              |
|                                 | Taka-matsu     | 2014 | Wnt3a    | Human   | 2D      | -                         | Wnt3a                           | In medium                      | Inhibiting canonical signaling prevents cartilage degradation                   | N/A           | N/A          | Negative       | PG production decreased                                                     |
| NP cells                        | Pizzute        | 2018 | Wnt3a    | Human   | 3D      | -                         | Wnt3a                           | Overexpression                  | Not confirmed                                                                 | Increase      | N/A          | Neutral        | Enhanced proliferation, no difference in chondrogenic markers or PG production |
|                                 | Hiyama         | 2011 | Wnt3a    | Rat     | 2D      | -                         | Wnt3a                           | 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    | Rat     | 2D      | -                         | Wnt3a                           | 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    | Human   | 3D      | -                         | FGF2                            | Cells separated based on expression of ROR2 receptors                         | N/A           | Increased    | Positive        | ROR2+ cells had an increased PG production                                  |
|                                 | Church         | 2002 | Wnt5a    | Chicken | 3D      | -                         | Wnt5a                           | Overexpression                  | Different non-canonical pathways                                              | Neutral       | N/A          | Positive        | Increased PG production and number of nodules, decreased expression hypertrophic markers |
|                                 | Pei            | 2014 | Wnt5a    | Human   | 3D      | -                         | TGF-β3                          | Expression measured             | Not confirmed                                                                 | N/A           | N/A          | Positive        | Expression wnt5a high in substrates with greatest chondrogenic potential    |
|                                 | Hsu            | 2013 | Wnt5a    | Rat     | 3D      | -                         | TGF-β3                          | Expression measured             | Canonical signaling more activated on osteogenesic substrates while non-canonical more on chondrogenic substrates | N/A           | N/A          | Positive        | Expression wnt5a high in substrates with greatest chondrogenic potential    |

(Continued to the next page)
| Cell type                        | Author                  | Year | Wnt  | Addition during expansion | Species | Culture | Supplementation method or Wnt3a | Signaling pathway                                                                 | Proliferation | Cell potency | Chondrogenesis                  | Outcome summary                                                                 |
|---------------------------------|-------------------------|------|------|---------------------------|---------|---------|---------------------------------|---------------------------------------------------------------------------------|---------------|--------------|-----------------------------|--------------------------------------------------------------------------------|
| Embryonic stem cells (differentiated towards MSCs) | Gibson                  | 2017 | Wnt5a| bFGF                      | 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| -                          | 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 |
|                                 | Hosseni-Farahabadi     | 2017 | 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| -                          | 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| -                          | Rat     | 2D      | Overexpression                  | Not confirmed                                                                 | Neutral       | N/A          | Neutral                     | No difference in aggregcan expression                                                                 |
|                                 | Li                     | 2018 | Wnt5a| -                          | Rat     | 2D      | Overexpression                  | 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-dimensional; 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; Wnt-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.\textsuperscript{16} However, \textit{WNT5A} overexpression is beneficial for the redifferentiation of NP cells as shown by the upregulation of specific NP cell markers such as \textit{PAX1} and \textit{FOXF1} and matrix genes \textit{COL2A1} and \textit{ACAN}.\textsuperscript{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\textsuperscript{57}), is significantly increased after \textit{WNT5A} overexpression\textsuperscript{48} and lost upon lentiviral knockdown of \textit{WNT5A}, as is the upregulation of the NP cell markers and matrix genes.\textsuperscript{16}

**CHONDROCYTES**

In chondrocytes, the effects of \textit{wnt3a} are described repeatedly to stimulate chondrogenic dedifferentiation.\textsuperscript{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 \textit{wnt3a} appears to upregulate proliferation rate.\textsuperscript{13,58} This could suggest that \textit{wnt3a} inhibits the maturation state of chondrogenesis and instead gives rise to a more potent cell phenotype.

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

**EMBRYONIC STEM CELLS**

In ESCs, \textit{wnt3a} is mainly reported to benefit expansion to generate a large number of chondrogenic potent cells. Moreover, \textit{wnt3a} is stated to inhibit chondrogenesis, and instead induces cells to adopt a more immature phenotype.\textsuperscript{26,65} Work by Tanaka et al.\textsuperscript{65} demonstrated that \textit{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\textsuperscript{26} found that when first supplying the cells with BMP4 for 5 days and thereafter replacing BMP4 for \textit{wnt3a}, chondrogenic potential could be optimized. Thus, suggesting that BMP4 is beneficial at the onset of differentiation while \textit{wnt3a} acts as a late inducer of chondrogenesis, indicating that the crosstalk of BMP4 and \textit{wnt3a} might be crucial for the chondrogenic differentiation of ESCs.

No work was identified that applied \textit{wnt5a} directly onto ESC for chondrogenic differentiation. However, Gibson and colleagues\textsuperscript{66} have applied \textit{wnt5a} supplementation with pellet cultures of ESCs that were predifferentiated to take on a MSC phenotype. Similar effects were reported by \textit{wnt5a} as observed with \textit{wnt3a}\textsuperscript{46} \textit{viz.} promoting chondrogenesis and limiting hypertrophy. Chondrogenic markers \textit{SOX9} and \textit{COL2A1} increased whereas the expression of hypertrophy markers; \textit{COL10A1} and \textit{ALP}, decreased. In the same study,\textsuperscript{66} the effect of BMP2 was found to upregulate early chondrogenic markers and proteoglycan production, attaining higher ratios than \textit{wnt5a}. However, BMP2 also significantly increased expression of hypertrophic markers. The sequential treatment of BMP2 and \textit{wnt5a} showed beneficial for inducing articular chondrocyte-like phenotypes, constituting initial chondrogenic induction via BMP2, followed by \textit{wnt5a} supplementation on the fifth day to maintain the chondrogenic phenotype and preventing hypertrophy.\textsuperscript{66}

**MESENCHYMAL STEM CELLS**

In MSCs, the chondrogenic effect of \textit{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, \textit{wnt3a} treatment lead to significantly higher proliferation rates.\textsuperscript{21,23,67} Nevertheless, \textit{wnt3a} does appear to have a negative effect on chondrogenesis according to two independent reports\textsuperscript{21,49} applying either overexpression in rat MSCs\textsuperscript{51} or supplementation in chicken embryo limb-bud MSCs.\textsuperscript{49}
Two studies 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 or highest WNT3A expression in the paper of Pei et al. 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 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. 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, however, addition of FGF2 during wnt3a stimulated monolayer culture abolished these beneficial effects. In the research of Fischer et al., overexpression of WNT3A in combination with BMP2 in murine embryo derived MSCs, resulted in significantly higher proteoglycan production. 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, 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. 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. 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, further emphasizing the role of wnt5a in early chondrogenic differentiation. Finally, Hsu and Huang 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. 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. 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 and cells 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. Nevertheless, IVD cells are commonly derived from compromised tissue sources presenting low cellular yield and reduced cell potency. Moreover, standard expansion of NP cells in vitro further reduces their overall potency. 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. 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
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 production57 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.

![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).](image-url)
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NP cell redifferentiation

+ Wnt5a

A

Dedifferentiation

+ Wnt5a

B

Chondrogenic differentiation

hypertrophic inhibition

+ Wnt5a

C

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, and immunomodulatory properties. Moreover, MSCs possess multipotent differentiation capabilities, including towards NP cell-like phenotypes, 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. Nonetheless, MSCs are being explored in clinical setting, and their primary data suggest MSC transplantation to be safe and able to provoke clinical improvements. 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. 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. Moreover, data suggest wnt5a interferes with hypertrophic maturation (Fig. 4G). 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\cite{38,39,40} and preculturing of MSCs under hypoxic conditions proved to be safe and promising in human clinical trials against IVD degeneration, 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.

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