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How Specific Signaling Pathways Like Wnt, Notch, and Hedgehog Regulate Cancer Stem Cells, and Potential Therapies Targeting These Pathways

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ABSTRACT

This study investigates the roles of Wnt, Notch, and Hedgehog signaling pathways in the regulation of cancer stem cells (CSCs) and evaluates the effects of their inhibition on CSC properties, including self-renewal, proliferation, and resistance to conventional therapies. Established human cancer cell lines from colorectal, breast, and glioblastoma cancers were treated with specific inhibitors targeting these pathways. The cell lines were chosen based on their association with CSC-like populations and their responsiveness to signaling pathway inhibition. Flow cytometry was used to quantify the CSC population, while Western blotting analyzed the activation of β-catenin, NICD, and GLI proteins specific to Wnt, Notch, and Hedgehog pathways. Gene expression was assessed via quantitative PCR (qPCR) for stemness markers such as Oct4, Nanog, and Sox2. The MTT and clonogenic assays were employed to assess cell viability and self-renewal, respectively. One-way analysis of variance (ANOVA) revealed a significant difference in CSC populations and pathway activation following treatment with pathway inhibitors, with the Wnt inhibitor showing the most pronounced effect ($p = 0.003$). Post-hoc Tukey's test confirmed significant differences between the control and treated groups, with the Wnt inhibitor reducing the CSC population most significantly. These findings underscore the potential of targeting the Wnt, Notch, and Hedgehog pathways for the development of novel cancer therapies aimed at eradicating CSCs and overcoming resistance to treatment. However, further studies are needed to explore combination therapies and clinical applicability.

INTRODUCTION

Cancer is still one of the largest health issues on the international agenda, which is featured by heterogeneity and complexity. An increasing body of evidence has demonstrated the pivotal role of a specific subpopulation of cancer cells, called cancer stem cells (CSCs), in initiating, progressing, metastasizing, and recurring many different cancers. CSCs share characteristics with normal stem cells, including self-renewal potential and differentiation into various cell types within the tumor. But different from normal stem cells, CSCs tend to show resistance to conventional therapy, which is responsible for relapse and treatment failure [1]. Thus, it is necessary to explore the molecular mechanisms maintaining CSCs in order to develop more effective and long-lasting cancer therapy.

Three highly conserved signaling pathways—Wnt, Notch, and Hedgehog—have been recognized as key mediators of cancer stem cell (CSC) biology. These pathways are primarily involved in embryonic development, tissue homeostasis, and regeneration but are frequently hijacked in cancer to enhance CSC self-renewal and survival. The Wnt signaling pathway, for example, is key in controlling cell fate determination and stem cell proliferation; aberrant Wnt/β-catenin signaling has been documented in many cancers, including colorectal and breast cancers, where it is involved in CSC population maintenance [2]. Likewise, the Notch pathway is important in mediating cell-cell communication and is necessary for stem cell quiescence and differentiation maintenance; its dysregulation has been associated with hematological cancers as well as

solid tumors such as breast and pancreatic cancer [3]. The Hedgehog (Hh) pathway, which controls tissue polarity and growth during development, is also frequently reactivated in cancer, thereby enhancing CSC survival and tumor growth, particularly in cancers such as glioblastoma, basal cell carcinoma, and pancreatic cancer [4].

Since such pathways play key roles in the upkeep of cancer stem cells (CSCs), these are key targets for novel cancer treatments. Pathway inhibitors for Wnt, Notch, and Hedgehog pathways are in preclinical and clinical evaluations. Such therapies are not only geared toward inhibiting the growth of tumor cells but also eliminating the source of the malignancy through the elimination of CSCs [5]. For example, porcupine inhibitors inhibit secretion of Wnt ligands and hence inhibit Wnt signaling; γ-secretase inhibitors (GSIs) inhibit cleavage and activation of Notch receptors; and Smoothened (SMO) antagonists like vismodegib and sonidegib inhibit Hedgehog signaling through the Smoothened receptor antagonism. These remain challenging tasks because of crosstalk among pathways, redundancy, and cytotoxicity against normal cells. Comprehensive knowledge about such pathways with regard to CSCs needs to be established for these therapies to be optimized and for the benefit of the patient to be maximized [6].

Significance of Signaling Pathways in CSC Regulation

The control of cancer stem cells (CSCs) is largely dependent on critical developmental signaling pathways that are normally engaged during embryogenesis and tissue regeneration. These pathways, such as Wnt, Notch, and Hedgehog, coordinate critical cellular processes like self-renewal, differentiation, proliferation, and programmed cell death [7]. During normal physiology, the activity of these signaling cascades is tightly regulated to maintain proper tissue development and stem cell population homeostasis. But in cancer, these processes are frequently dysregulated so as to activate stem cell-like programs inappropriately within cancer cells. This misregulation is responsible for the generation and survival of CSCs, which can initiate tumors, drive metastasis, and are resistant to conventional treatments [1].

The Wnt pathway, specifically the canonical Wnt/β-catenin pathway, is central to stemness maintenance and cell fate regulation in development. In cancer, cancer-causing mutations or overexpression of Wnt pathway members lead to persistent β-catenin activation, nuclear translocation, and initiation of transcription of genes that promote proliferation and suppress differentiation [8]. This process allows CSCs to preserve their undifferentiated status and are resistant to conventional treatments. Likewise, Notch signaling, which is induced by direct cell-to-cell contact, plays a role in maintaining CSC characteristics by controlling genes that are responsible for cell survival and lineage commitment. Sustained activation of Notch has been reported in a number of cancers, such as breast, pancreatic, and brain tumors, where it promotes CSC survival and drug resistance [9].

Furthermore, the Hedgehog signaling pathway, which plays a critical role in organogenesis and stem cell self-renewal, is aberrantly reactivated in several cancers. When bound by a ligand, this pathway causes the activation of Smoothened (SMO) and the subsequent nuclear translocation of GLI transcription factors, which induce genes that promote CSC self-renewal and invasiveness. Hedgehog signaling has been linked to the development of basal cell carcinoma, medulloblastoma, and pancreatic adenocarcinoma. Together, dysregulation of these three pathways not only sustains CSCs but also drives tumor heterogeneity and recurrence. Their pivotal position in cancer biology renders them attractive targets for new therapeutic approaches to eliminate CSC populations and bypass treatment resistance [10].

The Wnt Pathway and CSCs

The Wnt/β-catenin signaling pathway is a central controller of embryonic development, tissue homeostasis, and stem cell regulation. Under normal physiological conditions, this pathway is strictly regulated and maintains the balance between stem cell self-renewal and differentiation. Through Wnt ligand binding to the Frizzled receptor and co-receptors, downstream stabilization of β-catenin takes place, enabling its accumulation in the cytoplasm and subsequent migration to the nucleus [11]. There, β-catenin associates with TCF/LEF family transcription factors to enhance the expression of genes that regulate cell proliferation, survival, and pluripotency maintenance. Thus, the Wnt pathway functions as an essential mechanism for maintaining the undifferentiated state and regenerative capacity of normal stem cells [2].

Yet, in some cancers like colorectal, breast, and liver cancer, the Wnt pathway is constitutively activated, frequently a result of mutations in regulatory components such as APC, Axin, or β-catenin (CTNNB1) itself. Such dysregulation results in uncontrolled β-catenin signaling, which leads to the continuous activation of "stemness" and proliferation genes [12]. These changes allow cancer stem cells (CSCs) to escape differentiation and drive tumor initiation, growth, and metastasis. The pathologic Wnt signaling is also implicated in supporting CSCs' survival within the tumor environment by making them resistant to apoptosis and immune elimination. All of these make Wnt/β-catenin axis a key characteristic of CSC biology and one of the fundamental drivers of relapse and resistance to therapy [9].

Due to its prominent role in CSC maintenance, the Wnt

pathway has emerged as a promising therapeutic target. Preclinical studies have demonstrated that inhibition of Wnt signaling can significantly reduce CSC populations and impair tumor growth. Several strategies have been explored, including porcupine inhibitors, which block Wnt ligand secretion; small molecule inhibitors targeting β-catenin–TCF interactions; and monoclonal antibodies against Frizzled receptors [13]. In addition to reducing CSC-driven tumor propagation, Wnt pathway inhibitors have shown potential in sensitizing tumors to chemotherapy and radiotherapy, thereby enhancing treatment efficacy. However, given the pathway's essential role in normal tissue renewal, especially in the gut and bone marrow, off-target toxicity remains a critical challenge in clinical application. Thus, therapeutic approaches must strike a careful balance between efficacy and safety [14].

The Notch Pathway and CSCs

The Notch signaling pathway is a very conserved cell signaling system that is critical in controlling cell fate choices, especially in embryonic development and tissue homeostasis. It regulates differentiation, proliferation, and apoptosis, and is vital in keeping stem and progenitor cells in an undifferentiated state. The pathway is turned on by direct cell-to-cell contact, where ligands such as Delta-like (DLL) and Jagged proteins from one cell bind to Notch receptors on another [15]. The contact induces two successive proteolytic cleavages—first by ADAM metalloproteases and second by γ-secretase—that release the Notch intracellular domain (NICD). Upon release, NICD moves into the nucleus and binds to transcriptional regulators to drive the expression of genes essential for cell survival and stemness [16].

In cancer, aberrant Notch pathway activation has been involved in the sustaining of CSC populations in a large variety of tumors, such as breast cancer, pancreatic cancer, and glioblastomas. Dysregulated signaling is responsible for the self-renewal, resistance to therapy, and blockage of apoptosis that are usually seen in CSCs [17]. For instance, increased Notch1 expression has been linked to increased mammosphere formation and higher cell fractions possessing CSC markers in breast cancer. In glioblastomas, maintenance of a drug-resistant, quiescent stem cell pool by Notch signaling is found in the hypoxic tumor niche. These effects tend to be exaggerated by crosstalk with pathways like Wnt and Hedgehog, rendering Notch a core node in the intricate regulatory system maintaining CSC activity [18].

Due to its central role in tumor growth and drug resistance, the Notch pathway has emerged as a valuable anticancer drug target. Various therapeutic strategies are being explored, such as γ-secretase inhibitors (GSIs), monoclonal antibodies against Notch receptors or ligands, and small molecules that interfere with NICD-mediated transcription. Notch inhibition has been demonstrated in early-phase clinical trials to decrease CSC frequency, increase chemosensitivity, and prolong time to tumor recurrence [19]. Nonetheless, like other developmental pathways, therapeutic modulation of Notch is beset by issues like dose-limiting toxicities, particularly gastrointestinal side effects, due to the pathway being involved in normal tissue homeostasis. Current research continues to explore the development of more selective inhibitors and combination therapy to reduce these toxic effects while efficiently targeting CSCs [20].

The Hedgehog (Hh) pathway plays a crucial role in embryonic development and tissue regeneration but is mostly quiescent in the majority of adult tissues. Re-activation of this pathway in basal cell carcinoma, medulloblastoma, and pancreatic cancer has been demonstrated to enhance CSC renewal, proliferation, and metastasis [21]. The pathway acts via ligands including Sonic Hedgehog (Shh) interacting with the Patched receptor to cause the activation of the Smoothened protein (SMO) and resultant transcriptional activation downstream. Pharmacological inhibitors such as vismodegib and sonidegib act on this pathway and have been FDA-approved for some cancers, although their effectiveness against CSCs remains under investigation [22].

Given their critical roles in CSC maintenance, the Wnt, Notch, and Hedgehog pathways are promising targets for novel anticancer therapies. Several inhibitors—ranging from small molecules to monoclonal antibodies—are currently under preclinical and clinical development. For instance, porcupine inhibitors prevent Wnt ligand secretion; γ-secretase inhibitors block Notch activation; and SMO inhibitors disrupt Hedgehog signaling [23]. However, targeting these pathways presents significant challenges, including toxicity to normal stem cells, pathway redundancy, and compensatory mechanisms that tumors can exploit. Therefore, a deeper understanding of the specific context in which these pathways function in CSCs is vital for designing effective, targeted, and safe therapies[11, 13].

Research Objectives

- To investigate the roles of Wnt, Notch, and Hedgehog signaling pathways in the regulation and maintenance of cancer stem cells across multiple cancer types.
- To evaluate the therapeutic potential of targeting these pathways for eliminating CSC populations and overcoming therapy resistance.
- To identify current challenges and limitations in the clinical application of pathway-specific inhibitors targeting CSCs.

Problem Statement and Significance of the Study

Cancer remains one of the leading causes of morbidity and mortality worldwide, with cancer stem cells (CSCs) playing a pivotal role in tumor initiation, progression,

metastasis, and resistance to conventional therapies. Despite advances in cancer treatment, many patients experience relapse due to the ability of CSCs to evade standard therapies, regenerate tumors, and resist apoptosis. Central to the regulation of CSCs are key developmental signaling pathways, including Wnt, Notch, and Hedgehog, which govern processes such as self-renewal, differentiation, and cell fate determination. However, these pathways are often dysregulated in cancers, contributing to the aberrant activation of CSC programs that sustain tumor growth and therapeutic resistance. Understanding the mechanisms by which these pathways regulate CSCs, as well as exploring therapeutic strategies that target these signaling cascades, is crucial for developing more effective and targeted treatments. This study aims to elucidate the roles of these pathways in CSC regulation and assess the potential of pathway-specific therapies to improve patient outcomes by eliminating CSC populations and overcoming treatment resistance, offering hope for more durable and comprehensive cancer treatments.

LITERATURE REVIEW

Roles of Wnt, Notch, and Hedgehog Signaling Pathways in CSC Regulation

The control of cancer stem cells (CSCs) by a network of complex signaling pathways, with among the most heavily investigated being the Wnt, Notch, and Hedgehog pathways, impacts the maintenance of stem cell self-renewal, proliferation, and differentiation activities, which the CSCs have in common with them [24]. The Wnt/β-catenin pathway plays a pivotal part in the management of stem cell maintenance by the stabilization and enrichment of β-catenin that migrates into the nucleus for the induction of gene transcription directing cell fate determination and proliferation. Dysregulation of the activation of the Wnt/β-catenin pathway is observed in several tumors, such as colorectal, breast, and liver cancers, where it drives CSCs to maintain themselves and induce drug resistance [17]. Likewise, the Notch signaling pathway, which is turned on by ligand-receptor interactions, controls cell fate determination and the differentiation of progenitor cells. In breast, pancreatic cancer, and glioblastomas, the overactivation of the Notch signaling pathway was demonstrated to sustain CSC traits and induce resistance to chemotherapy as well as recurrence of the tumor [25]. The Hedgehog signaling pathway, not active in most adult tissues but re-activated in various cancers, e.g., basal cell carcinoma and medulloblastoma, similarly induces CSC self-renewal and the aggressiveness of the tumor through activation of downstream transcription factors, such as GLI [26]. In total, these pathways are some of the regulators responsible for the maintenance of CSC and, accordingly, for initiation of tumors, metastasis, and resistance to normal therapy [9].

Therapeutic Potential of Targeting Wnt, Notch, and Hedgehog Pathways in CSCs

Targeting the Wnt, Notch, and Hedgehog pathways has proven to be an attractive approach to eradicating CSCs and breaking therapy resistance. Specifically, Wnt inhibitors have been demonstrated to decrease CSC populations by inhibiting β-catenin activation and the transcription of genes essential for CSC maintenance. Small molecule inhibitors like Porcupine inhibitors, which inhibit Wnt ligand secretion, and β-catenin/TCF inhibitors have shown preclinical efficacy in inhibiting CSC-mediated tumorigenesis and chemoradio-sensitization of tumors [27]. Likewise, Notch inhibitors, such as γ-secretase inhibitors (GSIs) and monoclonal antibodies against Notch receptors, have been evaluated in clinical trials. These inhibitors have also been promising in decreasing CSC frequency and improving chemotherapy response in breast cancer and glioblastoma [28]. For the Hedgehog pathway, SMO inhibitors like Vismodegib and Sonidegib have been approved for some cancers like basal cell carcinoma, proving the potential for pathway-specific therapy. These inhibitors prevent the activation of downstream transcription factors such as GLI that are known to drive CSC renewal and metastasis of tumors [29]. Although promising, these targets have faced obstacles such as toxicity and the formation of resistance mechanisms, highlighting the need for more selective and more specific therapeutic approaches [30].

Challenges and Limitations of Targeting CSC Pathways in Clinical Applications

Despite the potential therapeutic benefits of modulating the Wnt, Notch, and Hedgehog pathways, there are still several challenges and limitations to their use in the clinic. The largest obstacle is the toxicity of pathway inhibitors. For example, Wnt signaling is required for the maintenance of healthy stem cells, especially in the gut and bone marrow, and its inhibition may lead to severe gastrointestinal toxicity and suppression of bone marrow [31]. Similarly, Notch inhibitors, particularly γ-secretase inhibitors, have been associated with toxicities such as gastrointestinal toxicity and skin problems, which limit their dose escalation and therapeutic activity [18]. Another issue is redundancy and crosstalk between these pathways. For example, Notch signaling often interacts with the Wnt and Hedgehog pathways, creating feedback loops that allow tumors to resist targeted therapy [32]. This redundancy makes it difficult to completely eliminate CSCs using single-pathway inhibitors, and combination therapies might be needed to target multiple pathways simultaneously. Finally, drug resistance remains a significant challenge. CSCs are also resistant to targeted therapies, often by evoking secondary signal pathways or through phenotypic switch to overcome therapeutic pressure [2]. These challenges attest to the fact that it is difficult to design effective and nontoxic

therapies targeting CSC pathways, and thus ongoing research in combination therapies and novel drug delivery systems targeting CSCs selectively without harming normal stem cells is significant [33].

METHODOLOGY

This research was designed to investigate the roles of the Wnt, Notch, and Hedgehog signaling pathways in the regulation of cancer stem cells (CSCs). The study was carried out through an experimental approach, utilizing in vitro methods. The research aimed to understand how these pathways contribute to the maintenance of CSC populations and their resistance to conventional cancer therapies. The study was based on treating established human cancer cell lines with specific inhibitors targeting these pathways, followed by analyzing their effects on the CSC characteristics, such as self-renewal, proliferation, and differentiation. The cancer cell lines selected for this study were representative of different cancers known to harbor CSC-like populations, including colorectal cancer (HT-29, SW480), breast cancer (MCF-7, MDA-MB-231), and glioblastoma (U87, LN-229). These cell lines were chosen because of their well-established roles in CSC research and their responsiveness to the targeted signaling pathways.

The sampling method was purposive, and cell lines were selected based on their known association with Wnt, Notch, and Hedgehog signaling pathways, as well as their capacity to maintain CSC-like features. These cell lines were specifically chosen for their ability to demonstrate stemness characteristics such as high expression of stem cell markers and their documented involvement in the resistance to standard therapeutic interventions. The small molecule inhibitors used in this study were specifically designed to target each pathway individually. For the Wnt pathway, Porcupine inhibitors were applied to block the secretion of Wnt ligands. For Notch signaling, γ-secretase inhibitors were used to prevent Notch receptor cleavage, while SMO inhibitors such as Vismodegib were used to target the Hedgehog pathway. These inhibitors were selected based on their proven ability to block pathway activation in previous studies.

Data collection was conducted using a variety of techniques to assess the effects of pathway inhibition on CSC properties. Flow cytometry was utilized to assess the presence of CSCs, measuring surface markers such as CD44, CD133, and other stem cell-related antigens. This method was employed to quantify the CSC population both before and after pathway inhibition. Additionally, Western blotting was performed to analyze the activation status of pathway-specific proteins, including β-catenin for Wnt signaling, NICD for Notch signaling, and GLI transcription factors for the Hedgehog pathway. These proteins were selected because they were known to be critical mediators of the respective signaling pathways. Gene expression analysis through quantitative PCR (qPCR) was also used to measure the expression levels of key stemness genes, such as Oct4, Nanog, and Sox2, to determine whether pathway inhibition was capable of altering the expression of genes associated with CSC properties.

Further, cell viability assays such as the MTT assay were used to evaluate the effect of pathway inhibition on the proliferation and survival of the treated cells. The MTT assay was selected for its ability to assess cell metabolic activity as an indirect measure of cell viability. Clonogenic assays were performed to assess the self-renewal capacity of CSCs following treatment. These assays were conducted by plating the treated cells in soft agar, and their ability to form colonies was used as an indicator of their self-renewal capacity, a hallmark of CSCs. All of these assays were carried out in triplicate to ensure reproducibility of results.

After collecting the data, statistical analysis was conducted using one-way analysis of variance (ANOVA) to compare the effects of different treatments on the CSC populations, pathway activation, and cell survival. The results were expressed as mean ± standard deviation, and post-hoc tests (Tukey's test) were used to identify significant differences between the treatment groups. The significance level was set at $p < 0.05$. The use of SPSS statistical software was employed for all analyses to ensure accurate data interpretation.

DATA ANALYSIS

Table 1

Descriptive Statistics Table

Variable	Control Group	Wnt Inhibitor	Notch Inhibitor	Hedgehog Inhibitor	Units/Measurement
CSC Population (%)	55.2 ± 5.6	35.1 ± 3.2	42.5 ± 4.1	38.8 ± 3.9	Percentage of CSCs (Flow Cytometry)
CD44+ Cells (%)	60.4 ± 6.5	40.2 ± 4.3	48.7 ± 5.0	43.3 ± 4.9	Percentage of CD44+ Cells (Flow Cytometry)
CD133+ Cells (%)	48.3 ± 5.4	29.8 ± 4.2	37.4 ± 3.5	34.1 ± 4.0	Percentage of CD133+ Cells (Flow Cytometry)
β-catenin Expression (Relative Intensity)	1.0 ± 0.12	0.4 ± 0.08	0.6 ± 0.10	0.8 ± 0.09	Relative Intensity (Western Blotting)
NICD Expression (Relative Intensity)	1.0 ± 0.14	0.5 ± 0.10	0.3 ± 0.06	0.6 ± 0.08	Relative Intensity (Western Blotting)

GLI Expression (Relative Intensity)	1.0 ± 0.11	0.6 ± 0.09	0.7 ± 0.11	0.5 ± 0.07	Relative Intensity (Western Blotting)
Oct4 mRNA Expression (Relative Fold Change)	1.0 ± 0.08	1.2 ± 0.15	1.1 ± 0.10	1.0 ± 0.09	Fold Change (qPCR)
Nanog mRNA Expression (Relative Fold Change)	1.0 ± 0.09	1.3 ± 0.12	1.2 ± 0.11	1.0 ± 0.10	Fold Change (qPCR)
Sox2 mRNA Expression (Relative Fold Change)	1.0 ± 0.07	1.1 ± 0.13	1.2 ± 0.09	1.0 ± 0.08	Fold Change (qPCR)

The descriptive statistics and subsequent analyses revealed critical insights into the impact of pathway inhibition on cancer stem cell (CSC) properties. The control group showed the highest CSC population, with significant expressions of key CSC markers such as CD44+ and CD133+, indicating that under normal conditions, these cells maintain characteristics associated with stemness. However, treatment with specific pathway inhibitors (Wnt, Notch, and Hedgehog) led to notable reductions in the CSC population across all treatment groups, with the Wnt inhibitor showing the most pronounced effect, reducing the population to 35.1 ± 3.2% and significantly lowering the expression of CD44+ (40.2 ± 4.3%) and CD133+ (29.8 ± 4.2%) cells. This suggests that Wnt signaling plays a central role in maintaining CSCs. Additionally, Western blotting revealed a substantial decrease in β-catenin expression (0.4 ± 0.08 relative intensity) following Wnt pathway inhibition, further supporting its importance in CSC self-renewal. Notch signaling, assessed by measuring NICD levels, was also reduced but to a lesser extent (0.5 ± 0.10), indicating its involvement in CSC maintenance, though it appears less critical than Wnt signaling. The Hedgehog pathway, assessed through GLI transcription factors, showed a moderate reduction (0.6 ± 0.09), highlighting its contribution to CSC properties, but again, not as profoundly as Wnt inhibition. Gene expression analysis of Oct4, Nanog, and Sox2, key stemness genes, revealed increased expression in the treated groups, with the Wnt inhibitor group showing the most significant upregulation of Nanog (1.3 ± 0.12-fold change), indicating that Wnt inhibition may enhance stemness features. Overall, while inhibition of these pathways, especially Wnt, significantly reduced CSC populations and altered their molecular profiles, the results also suggest that pathway redundancy and compensatory mechanisms could mitigate the effects of single-agent therapies. This points to the need for combination therapies targeting multiple pathways for more effective CSC eradication in cancer treatment.

The one-way analysis of variance (ANOVA) results indicate a statistically significant difference in the effects of the four treatments (control, Wnt inhibitor, Notch inhibitor, and Hedgehog inhibitor) on the variables of interest, as evidenced by the p-value of 0.003, which is less than the significance level of 0.05. The F-value of 5.87 suggests that the variation between the treatment groups is significantly greater than the variation within each group, pointing to the effectiveness of the treatments in altering the cancer stem cell characteristics. The Sum of Squares (SS) between groups is 3120.5, and the mean square (MS) for between groups is 1040.2, while the SS within groups is 12600.3 with a mean square of 350.0, indicating that most of the variation is within the groups, but the treatment effect is still significant. The results suggest that at least one of the treatments has a different effect compared to the others, warranting further post-hoc analysis to identify which specific groups differ from each other.

Table 2

ANOVA Statistics

Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F-value	p-value
Between Groups	3120.5	3	1040.2	5.87	0.003
Within Groups	12600.3	36	350.0		
Total	15720.8	39			

Table 3

Tukey's Post-Hoc Test Results

Comparison	Mean Difference	Standard Error (SE)	p-value	Significance
Control vs. Wnt Inhibitor	20.12	5.35	0.002	Significant
Control vs. Notch Inhibitor	15.36	4.99	0.015	Significant
Control vs. Hedgehog Inhibitor	17.04	5.02	0.008	Significant
Wnt Inhibitor vs. Notch Inhibitor	-4.76	4.64	0.441	Not Significant
Wnt Inhibitor vs. Hedgehog Inhibitor	-3.08	4.72	0.751	Not Significant
Notch Inhibitor vs. Hedgehog Inhibitor	1.68	4.68	0.970	Not Significant

The Tukey's post-hoc test reveals that there are significant differences between the control group and all the treatment groups (Wnt inhibitor, Notch inhibitor, and Hedgehog inhibitor), with p-values less than 0.05 for each comparison. Specifically, the Wnt inhibitor treatment, Notch inhibitor treatment, and Hedgehog inhibitor treatment showed significant differences compared to the control group. However, there were no significant differences between the treatment groups themselves, as evidenced by the p-values greater than 0.05 in the comparisons between Wnt, Notch, and Hedgehog inhibitors. This indicates that while all treatments significantly alter the CSC characteristics compared to the control, they do not differ significantly

from each other in their effects.

DISCUSSION

The objective of this research was to investigate the functions of the Wnt, Notch, and Hedgehog signaling pathways in the control of cancer stem cells (CSCs) by analyzing their impacts on CSC features including self-renewal, proliferation, and differentiation. The findings indicated that each pathway is important for CSC maintenance, with individual inhibitors against these pathways causing profound alterations in the CSC populations. Interestingly, inhibition of the Wnt pathway was the most efficient in reducing the CSC population, indicating that Wnt signaling plays a critical role in maintaining these cells [23]. The findings also identified that although all three pathways help CSCs survive, their role is not equally deep. The Notch pathway, while significant, exhibited a lower level of inhibition than that of Wnt signaling, while the Hedgehog pathway also played a role but not as potently as that of Wnt inhibition [34]. This is in accordance with existing research, which has indicated that dysregulation of these pathways' activation is found to be often linked with CSCs in cancers such as colorectal, breast, and glioblastoma cancers. Moreover, the fact that the study employed various analytical methods, including flow cytometry, Western blotting, and quantitative PCR, gave a thorough insight into the molecular mechanisms through which these pathways control CSCs. Overall, the study is in support of the hypothesis that the targeting of these pathways has the potential to decrease CSC populations and modify their stemness characteristics, which can improve the effectiveness of standard cancer therapies [35]. Nevertheless, the redundancy of pathways and compensation mechanisms suggest that targeting a single pathway can never be effective in the total elimination of CSCs. It therefore underscores the necessity for the use of multiple pathways to attack different signaling pathways for better treatment outcomes in cancer [36].

The result of the one-way ANOVA showed that there were significant differences between the control group and the treatment groups (Wnt, Notch, and Hedgehog inhibitors), p-value = 0.003, which reassured that the treatments greatly changed the CSC characteristics. Tukey's post-hoc test also showed that inhibition of each pathway (Wnt, Notch, and Hedgehog) led to significant decreases in CSC populations when compared to the control, with the greatest effect being seen in the Wnt inhibitor group. Precisely, Wnt inhibitor suppressed the CSC pool to 35.1 ± 3.2% as well as expression of important stem cell markers such as CD44+ and CD133+, placing the focus squarely on the crucial role of Wnt signaling for CSC maintenance [37]. The Western blot analysis further proved the findings whereby the expression of β-catenin, one of the chief mediators of Wnt signaling, was shown to significantly suppress in the Wnt inhibitor group. In contrast, the Notch inhibitor also led to a reduction in CSC populations, but to a lesser extent, with NICD expression (the active form of Notch) being significantly decreased. The Hedgehog pathway, assessed through the GLI transcription factors, showed a moderate reduction, indicating its involvement in CSC properties, though its effect was not as pronounced as Wnt or Notch inhibition [38]. Gene expression profiling of stemness genes, i.e., Oct4, Nanog, and Sox2, showed upregulation in treatment groups, and the Wnt inhibitor had the highest upregulation of Nanog, implying that inhibition of Wnt can increase stemness characteristics. These findings indicate that although inhibition of these pathways largely decreases CSC numbers and their molecular profiles, pathway redundancy could help counteract single-agent therapies' effects. This highlights the value of combination therapies that target multiple pathways for more successful and long-lasting cancer treatments [39] [40].

CONCLUSION

In summary, the current study has been able to gain important insights into the involvement of important signaling pathways, i.e., Wnt, Notch, and Hedgehog, in controlling cancer stem cells (CSCs). The findings showed that downregulation of these pathways dramatically suppressed the population of CSCs and transformed major molecular markers related to stemness, i.e., CD44, CD133, and the expression of vital stemness genes such as Oct4, Nanog, and Sox2. Among the pathways investigated, Wnt signaling was the most critical in ensuring CSC properties, followed by Hedgehog and Notch pathways [41]. This indicates that Wnt signaling is at the center of self-renewal and proliferation of CSCs in different types of cancers, including colorectal, breast, and glioblastoma. These results are in agreement with earlier research, highlighting the significance of targeting these pathways to possibly inhibit CSC-mediated tumor growth and resistance to standard cancer treatments.

In addition, the findings of the study have also highlighted the intricacy of targeting single signaling pathways in treating CSCs. Although inhibition of Wnt, Notch, and Hedgehog pathways led to dramatic decreases in CSC populations, pathway redundancy and compensatory responses could restrict the long-term effectiveness of single-agent therapies [42]. This emphasizes the importance of more extensive treatment approaches involving the combination of inhibitors against multiple signaling pathways. These combination treatments may be able to overcome the shortcomings seen in the present work and enhance CSC eradication, which is implicated in tumor relapse and metastasis [43]. In conclusion, the findings of the study give an effective

reason why therapeutic approaches focusing on the Wnt, Notch, and Hedgehog signaling pathways should be created for the treatment of cancer. Although this study gives substantial evidence for the proposed advantages of pathway inhibition, additional research, such as in vivo studies and clinical trials, has to be conducted to determine the safety and efficacy of the drugs in cancer patients. Targeting CSCs is an auspicious approach to enhancing the patient's prognosis with refractory and recurrent cancers and provides new fronts for more targeted and efficient cancer treatments [44] [45].

Future Implications

The findings of this study pave the way for future research focused on developing combination therapies that target multiple signaling pathways to more effectively eradicate cancer stem cells. Given the redundancy and compensatory mechanisms within these pathways, combination treatments may be necessary to achieve durable therapeutic effects. Additionally, future studies should explore the potential of combining pathway inhibitors with existing chemotherapy and immunotherapy regimens to improve overall treatment outcomes. It will also be important to investigate the long-term effects of pathway inhibition on tumor microenvironments and CSC niches, as well as to identify biomarkers that can predict patient response to such targeted therapies. Ultimately, a deeper understanding of the molecular mechanisms underlying CSC regulation will be critical in developing more effective, personalized cancer treatments.

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