Masnoon et al., *BioImpacts*. 2026;16:33404
doi:10.34172/bi.33404
https://bi.tbzmed.ac.ir/

Naphthoquinones mediate differentiation of human umbilical cord derived mesenchymal stem cells into insulin producing cells through regulation of Wnt and BMP pathways

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Article Info

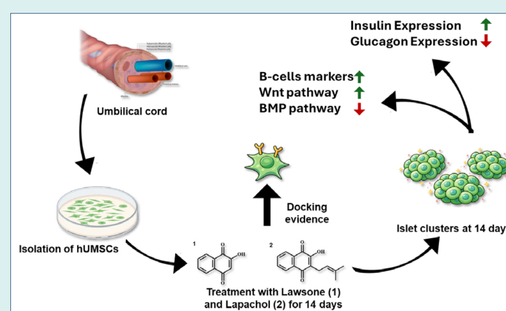
Article Type:
Original Article

Article History:
Received: 25 Dec. 2025
Revised: 11 May 2026
Accepted: 12 May 2026
ePublished: 7 Jul. 2026

Keywords:
BMP
Differentiation
Insulin producing cells
MSCs
Naphthoquinone
Wnt

Abstract

Introduction: Diabetes mellitus (DM) being a chronic metabolic disorder, causes a major concern for the healthcare system. Among different types, type 1 DM (T1DM) results in the destruction of insulin-producing pancreatic β cells, mediated by the immune system. Studies have demonstrated that human umbilical cord derived mesenchymal stem cells (hUMSCs) exhibit great potential to regenerate β-cells. Moreover, in order to enhance the regenerative potential of MSCs, several strategies are being utilized, including preconditioning with bioactive compounds. Among these, naphthoquinones can be used for MSC preconditioning in order to augment their therapeutic potential for β-cell regeneration, as these compounds possess anti-inflammatory and anti-diabetic properties.



Methods: hUMSCs were isolated, characterized, and treated with non-cytotoxic concentrations of lawsone, lapachol, or their combination. The preconditioned cells were subsequently analyzed for pancreatic β-cell differentiation at gene and protein levels. The study also explores the role of Wnt and BMP signaling pathways during the differentiation process through gene expression analysis. Binding patterns of these compounds with their respective receptors were analyzed using *in silico* studies.

Results: Gene expression profiling showed overexpression of pancreatic β-cell–specific markers in the Law+hUMSC group, whereas downregulation of Neurogenin-3 (NGN3) was observed in all treatment groups. Immunocytochemical analysis also showed enhanced expression of insulin in Law+hUMSCs, relative to other groups. Transcriptional analysis of the wingless/integrated (Wnt) and bone morphogenetic protein (BMP) pathways showed increased Wnt and decreased BMP expression across all treatment groups. *In silico* analyses showed that the binding patterns of lawsone or lapachol with frizzled (FZD) and activin-like kinase 1 (ALK1) receptors share comparable sequence similarity, facilitating their binding to these receptors and regulating downstream Wnt and BMP signaling.

Conclusion: The study concludes that the regulatory role of lawsone and lapachol can be exploited for preconditioning of MSCs for improved pancreatic β-cell differentiation.

Introduction

Diabetes is a chronic metabolic disorder defined by defective insulin production and/or resistance, resulting in persistent hyperglycemia. The prevalence of diabetes has been constantly on the rise during the past decades, which highlights its growing public health burden across the globe.[1] It is now predicted to be ranked among the world's leading causes of mortality by 2030. Moreover, it is estimated that by 2040, diabetes will affect 642 million individuals worldwide. Diabetes is primarily classified into type 1, type 2, and gestational forms. Among them, type 1 diabetes mellitus (T1DM) accounts for about 9.5% of all cases. T1DM develops because of an autoimmune response resulting in pancreatic β-cell destruction.

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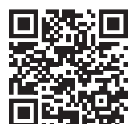

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Research Highlights

What is the current knowledge?

- Lack of standardized single-step differentiation approaches limits the reproducibility of MSC-derived IPC generation.
- Insulin expression in MSC-derived IPCs is often low, heterogeneous, and insufficient for functional applications.

What is new here?

- A single-molecule preconditioning strategy using lawsone/lapachol biases hUMSC fate toward a β-cell like phenotype.
- Simultaneous activation of Wnt signaling and suppression of BMP signaling promotes coordinated β-cell lineage commitment.

Individuals with T1DM depend on exogenous insulin for the management of hyperglycemia, however, diabetes associated complications cannot be prevented. In recent years, β-cell replacement through islet transplantation has emerged as a promising treatment option, however, limited availability of islet donors and constant immunosuppression is a major obstacle.[2]

The replacement of degenerated β-cells through regenerative therapies may provide hope for treating not only T1DM but also its associated complications.[3] Many studies have reported positive outcomes of these cell-based therapies. Embryonic stem cell (ESC)-derived insulin producing cells (IPCs) have shown remarkable hypoglycemic effect in streptozotocin (STZ)-induced diabetic mice.[4] Similarly, studies reported successful differentiation of mice ESCs into insulin producing cells using induction media and other factors.[5-7] Protocols for the differentiation of human ESCs (hESCs) toward the pancreatic endocrine lineage were established based on these findings.[8] Despite promising results, the application of hESC-derived IPCs in preclinical and clinical settings remains ethically debatable.[9] MSCs, on the other hand, are the adult, fibroblast-like non-hematopoietic multi-potent cells, having remarkable plasticity and less ethical concerns. Furthermore, the use of MSCs for therapeutic applications is preferred mainly due to their immunomodulatory activities. *In vitro* differentiation of MSCs into IPCs has also been extensively investigated using various protocols.[10-13] A study demonstrated the differentiation of human adipose tissue MSCs (hAD-MSCs) into IPCs using conditioned medium augmented with albumin, fibroblast-growth factor, and antibiotics in the presence of collagenase I. These extrinsic factors successfully differentiated hAD-MSCs into IPCs under *in vitro* conditions.[14] Similarly, a two-step protocol was shown to differentiate hAD-MSCs into IPCs using low-glucose DMEM, nicotinamide and β-mercaptoethanol followed by high-glucose DMEM, β-mercaptoethanol, exendin-4, and nicotinamide.[15]

Although naphthoquinones have been well-documented for antidiabetic and β-cell protective effects *in vivo*, their role as direct molecular modulators of lineage-specific signaling pathways during MSC differentiation remains unexplored. To date, no study has investigated a single small-molecule intervention to study MSC differentiation into pancreatic β-cell lineage[16] as a potential therapeutic strategy for the management of β-cell damage in T1DM.

In this study, two compounds from the naphthoquinone class, lawsone and lapachol were selected based on their quinone-based redox-active structures to precondition hUMSCs. Naphthoquinones are known for their diverse biological activities, i.e., anti-inflammatory, antioxidant, anticancer, and neuroprotective effects. They have been reported previously for the reversal of T1DM in mice [17] and/or rats.[18, 19] MSCs preconditioned with lawsone, have also been demonstrated for pancreatic β-cell regeneration potential in STZ-induced diabetic rats.[19] Considering the bioactive properties of naphthoquinones, this study is designed to evaluate the capability of hUMSCs, pretreated with lapachol, lawsone, or their combination, to differentiate into IPCs under controlled *in vitro* environment. The study also focused on evaluating the role of preconditioned cells in modulating Wnt and BMP signaling pathways during MSC commitment to IPCs. Furthermore, the study determined the binding pattern of lawsone or lapachol with Wnt and BMP receptors through *in silico* approach. The strategy to develop IPCs may serve as the treatment modality for the management of T1DM and its associated systemic complications.

Materials and Methods

Materials

The reagents/materials used in the study are as follows: Antibodies: anti-CD29, anti-CD117/c-kit, anti-vimentin (Sigma-Aldrich, USA); anti-CD90 (Cedarlane CELLutions Biosystems, Canada); anti-CD45 (BD Pharmingen, USA); anti-F-actin, Alexa fluor 488 and 546 goat anti-mouse IgG (Santa Cruz Biotechnology Inc., USA); anti-stro1 antibody, TRIzol reagent, anti-insulin, anti-glucagon, Alexa fluor 546 rat anti-rabbit IgG (Invitrogen, USA); Bovine serum albumin (BSA) (Merck, Millipore, USA); BrightGreen 2X qPCR MasterMix (Applied Biological Materials Inc., Canada); DAPI (MP biomedical Inc, USA); Dulbecco's modified eagle medium (DMEM), penicillin/streptomycin, Trypsin EDTA (Gibco, USA); DMSO, lawsone, lapachol, MTT, formaldehyde (Sigma-Aldrich, USA), fetal bovine serum (FBS), sodium pyruvate (Biowest, USA); RevertAid First Strand cDNA synthesis kit, Triton X-100 (Invitrogen, USA); Ethylenediaminetetraacetic acid (EDTA), Ethanol (Thermo Fisher Scientific , USA); Tween 20, Trypan Blue (MP biomedical, Inc, USA).

Ethical statement and sample collection

This study protocol was reviewed and approved by the Institutional Ethics Committee (IEC) at International Center for Chemical and Biological Sciences (ICCBS) with the assigned protocol number, "ICCBS/IEC-037-

HT-2018/Protocol/1.0". Cord tissue samples were obtained from healthy donors undergoing full-term cesarean deliveries at the Zainab Panjwani Memorial Hospital, Karachi, after obtaining their written informed consent. Collected samples were immediately placed in sterile glass containers (0.5% EDTA in PBS) and transported under cold/aseptic conditions to the cell culture laboratory at the ICCBS for processing.

Processing of umbilical cord tissue and explant culture

Cord tissues were processed under aseptic conditions under biosafety cabinet using mechanical/explant method, as previously described.[18] In brief, the cord tissue was rinsed with PBS to remove residual debris and blood clots. Explants of approximately 2-3 mm in size were transferred into T-75 culture flasks having DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. The culture flasks were kept at 37 °C in the 5% CO_2 incubator. Culture medium was refreshed every 24-72 h to maintain optimal growth conditions. After 10-15 days, cells migrated out of the explants, adhered to the flask surface, and started to proliferate. At around 80–90% confluence, the cells were sub-cultured to subsequent passages using trypsin EDTA. For all experiments, MSCs of passages 3 -5 were used.

hUMSC characterization

hUMSCs were characterized using immunocytochemistry and tri-lineage differentiation as reported elsewhere.[19]

Immunocytochemistry

Presence of MSC surface markers was analyzed via immunocytochemistry. hUMSCs were cultured on sterile cover slips placed in a 24-well plate and kept in a CO_2 incubator for 24 h. Cells were fixed using 4% paraformaldehyde solution for 10 min at room temperature and subsequently washed with PBS. Triton X-100 (0.1%) was used to permeabilize the cells for 10 min, followed by washing with PBS. To prevent non-specific binding, blocking solution (2% BSA) was added to each well for 30 min at 37°C. Primary antibodies against CD90, Stro1, Vimentin, or CD45 were added to the corresponding wells at a dilution of 1:100 and incubated overnight at 4 °C. Next day, the primary antibody solutions were removed, and the cells were rinsed with PBS. Alexa Fluor 546 goat anti-mouse secondary antibody (1:200) was added to each well for 1 h at 37 °C. Nuclei were stained with 0.5 µg/mL DAPI for 10 min. Cells were washed five times with PBS. The cover slips were mounted with fluorescent mounting medium, and analyzed under a fluorescent microscope (Eclipse NiE, Nikon).

Tri-lineage differentiation potential

hUMSCs were cultured in six-well plates under optimized *in vitro* growth conditions. Upon reaching full confluence, the basal culture medium was replaced with lineage-specific i.e. osteogenic, chondrogenic, or adipogenic induction medium, and maintained for 21 days. MSC differentiation was confirmed with Alizarin Red S, Alcian Blue, and Oil Red O staining, respectively. Representative images were acquired under a bright-field microscope (Eclipse NiE, Nikon).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cytotoxic effects of lawsone, lapachol, or their combination were evaluated at different concentrations by MTT assay as previously reported.[18] Briefly, hUMSCs were cultured in a 96-well plate and kept for incubation at 37 °C for 24 h. Different concentrations of each compound i.e., lawsone (5, 10, 25, 50 and 75 µM), lapachol (5, 10, 25, 50 and 75 µM), and combination (5, 10, and 25 µM of each compound) were added to the assigned wells and incubated for 24 h at 37 °C. Each concentration was tested in triplicate and untreated cells were used as control. Next day, MTT dye was added to each well for 4 h. MTT dye was removed, Dimethyl sulfoxide (DMSO) was added, and analyzed at an absorbance of 570 nm via a spectrophotometer (Multiskan Go; Thermo Fisher Scientific).

In vitro differentiation of hUMSCs into insulin producing cells (IPCs)

hUMSCs were categorized into four groups to observe their differentiation toward IPCs, as follows:

- Group 1: Untreated hUMSCs (Control)
- Group 2: Lawsone treated hUMSCs (Law + hUMSCs)
- Group 3: Lapachol treated hUMSCs (Lap + hUMSCs)
- Group 4: Lawsone and Lapachol treated hUMSCs (Law + Lap + hUMSCs)

hUMSCs were treated with the optimized concentration *i.e.* 10 µM of lawsone, lapachol, and their combination (Law + Lap) separately, in serum-free DMEM for 24 h.[18] The following day, compound containing medium was replaced with 10% FBS supplemented DMEM and cells were incubated at 37 °C for further 7 days. The same protocol was repeated for another week. After a two-week treatment, the cells were trypsinized for gene expression analysis.

Gene expression analysis

RNA isolation and cDNA synthesis

RNA was extracted from control and treatment groups using Trizol method according to manufacturer's instructions. The concentration and purity of RNA was determined at 260 nm *via* NanoDrop (Thermo Fisher Scientific,). Complementary DNA (cDNA) was synthesized using the RevertAid cDNA synthesis kit, following the instructions provided by the manufacturer.

Quantitative polymerase chain reaction (qPCR)

qPCR was performed to analyze the expression of pancreatic β-cell markers. Furthermore, genes specific to the Wnt and BMP pathways were also evaluated. The reactions were performed in triplicate, starting with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 58 °C for 1 min. The Ct values were used to calculate the fold change and glyceraldehyde-3-phosphate

dehydrogenase (*GAPDH*) was used as the standard internal control to normalize the expression of genes. The primer sequences for the targeted genes are provided in Table S1 (Supplementary file 1).

Protein expression analysis
Immunocytochemistry was performed to analyze the expression of insulin and glucagon in all the experimental groups, as mentioned above. The cells were visualized under a fluorescent microscope (Eclipse NiE, Nikon).

Statistical analysis
The experimental data were analyzed using IBM-SPSS statistics software. The values are reported as mean ± SEM, with an observation number (n) = 3 and a significance threshold of $P < 0.05$ ($P < 0.05$*, $P < 0.01$**, and $P < 0.001$***). The analysis utilized one-way ANOVA for multiple group comparisons with a post hoc Bonferroni test.

In silico analysis
Computational analyses were conducted to evaluate the molecular binding interactions of lawsone and lapachol with signaling receptors of the Wnt and BMP pathways, i.e., FZD and ALK1, respectively. Three-dimensional (3D) molecular structures of lawsone and lapachol were retrieved from the PubChem database. The structure of FZL protein was obtained from the RCSB database (PDB ID: 7EVW), whereas ALK-1 structure was created *via* SWISS modeling, using the ALK-1 template from the RCSB database (PDB ID: 7YRU). The models underwent three iterations of refining using Gibb's free energy and evaluated using MolProbity. The final versions of FZL and ALK-1 were subjected to blind docking with lawsone and lapachol using CB-dock using default parameters.

Results
hUMSC culture and passaging
After about 10-15 days of culture, hUMSCs moved out of the explants and started to adhere to the flask surface. The adherent cells multiplied and formed monolayer, exhibiting fibroblast-like appearance (P0). Once, the cells reached 80-90% confluence, they were sub-cultured to subsequent passages for continued expansion (Fig. 1A).

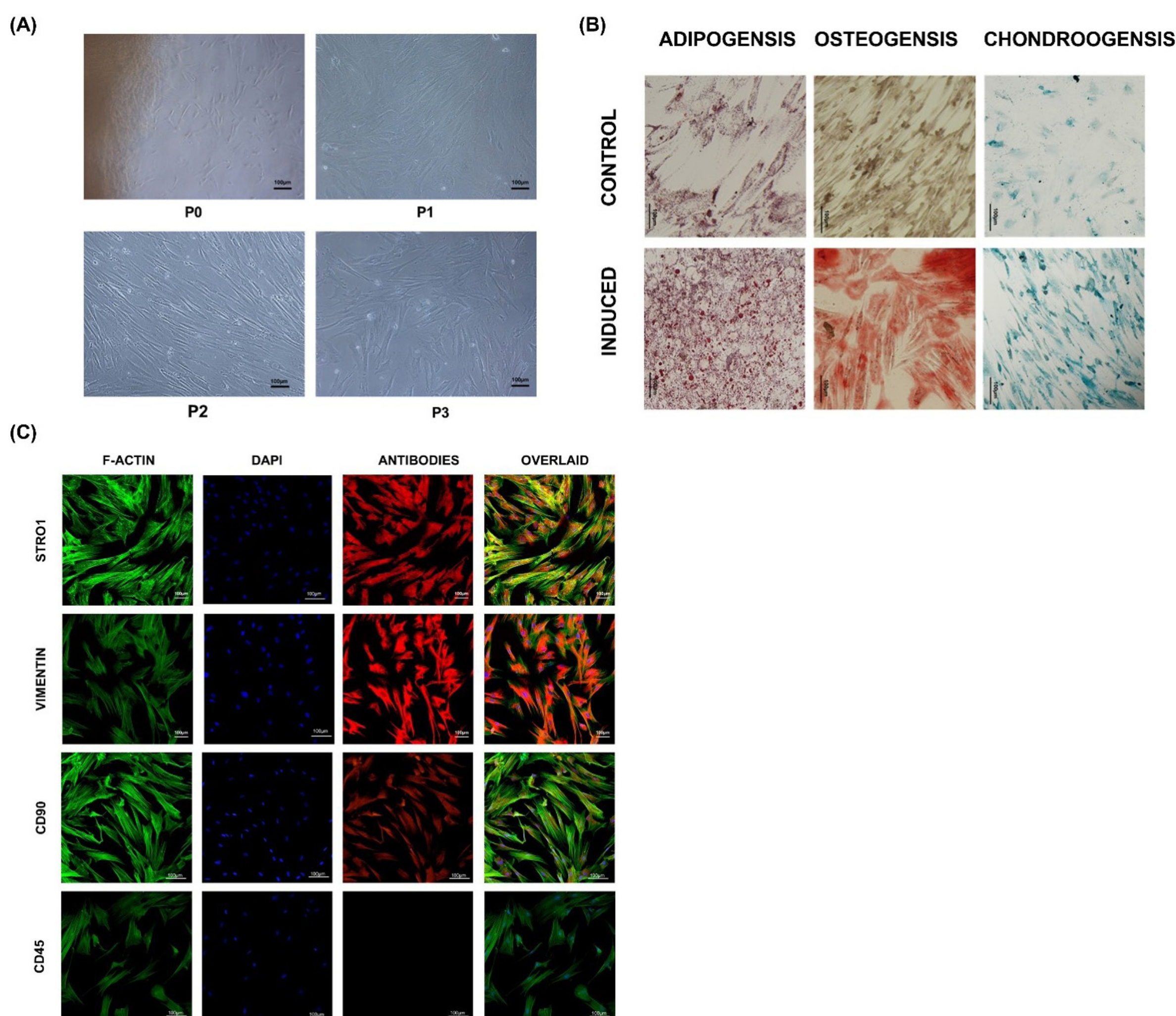

Fig. 1. Characterization of hUMSCs: **(A)** Morphological characteristics of hUMSCs at different passages, P0 - P3. **(B)** Tri-lineage differentiation of hUMSCs; Adipocytes (stained with Oil Red O), Osteocytes (stained with Alizarin Red), and Chondrocytes (stained with Alcian Blue). **(C)** hUMSCs demonstrating positive expression of MSC-specific markers (Stro1, Vimentin, and CD90), whereas, hematopoietic marker i.e., CD45 was not expressed, indicated by immunocytochemical analysis. Nuclei were stained with DAPI and cytoskeleton was stained with F-actin. All images were taken under fluorescent microscope.

hUMSC characterization

Immunocytochemistry

Immunocytochemical analysis of hUMSCs showed positive expression of Vimentin, Stro1, and CD90, specific to MSCs, while the hematopoietic marker CD45 was not detected (Fig. 1B).

Tri-lineage differentiation

Treated hUMSCs showed osteogenic, chondrogenic and adipogenic differentiation after 3 weeks in the lineage specific induction media, with distinct characteristics *i.e.*, mineral deposition, proteoglycans and oil droplets, respectively (Fig. 1C).

Differentiation of hUMSCs

Treatment of hUMSCs with the non-cytotoxic concentration of lawsone, lapachol (10 µM each), or their combination in serum free DMEM, showed differentiation toward IPCs after 14 days. The treated cells showed the formation of islet-like clusters (Fig. 2).

Gene expression analysis

qPCR analysis was performed to check the expression of genes specific to β-cells, and Wnt and BMP pathways. Law + hUMSCs showed significant upregulation of pancreatic β-cell markers, *i.e.*, insulin (*INS-1)*, MAF bZIP transcription factor A 1 (*MAFA1)*, pancreatic and duodenal homeobox 1 (*PDX1)*, forkhead box A2 *(FOXA2),* and NKX6 homeobox 1 *(NKX6.1)*, compared to the untreated control. However, the expression of these markers was found to be slightly higher but statistically non-significant in both Lap + hUMSC and combined treatment groups. Furthermore, the expression of *NGN3* was found to be reduced in all the treated groups as compared to the control group (Fig. 3).

Transcriptional dynamics of the Wnt pathway in lawsone- or lapachol-treated hUMSC groups revealed increased expression of Wnt mediators, including Wnt family member 2 (*WNT2*), catenin beta 1 (*CTNNB1*, encoding β-catenin), MYC proto-oncogene (*c-MYC*), Jun proto-oncogene (*c-JUN*), and cyclin D1 (*CCND1*). Conversely, the expression of Wnt pathway inhibitors, glycogen synthase kinase 3 beta (*GSK3β*) and axin 1 (*AXIN1*), was reduced compared to the control group (Fig. 4). Furthermore, expression of BMP pathway mediators, such as inhibitor of DNA binding 1 (*ID1*) and members of the Suppressor of Mothers Against Decapentaplegic (SMAD) family (*SMAD1*, *SMAD5*, *SMAD6*, and *SMAD7*), was consistently reduced in all treatment groups compared to the control (Fig. 5).

Protein expression analysis

Immunocytochemical analysis revealed the expression of insulin in each treated group, compared to control group (Fig. 6A). Law + hUMSCs showed the highest expression of insulin, compared to Lap + hUMSCs and the combined treatment groups (Fig. 6C). Furthermore, the differentiated hUMSCs did not show the expression of glucagon, an α-cell specific marker (Fig. 6B, D).

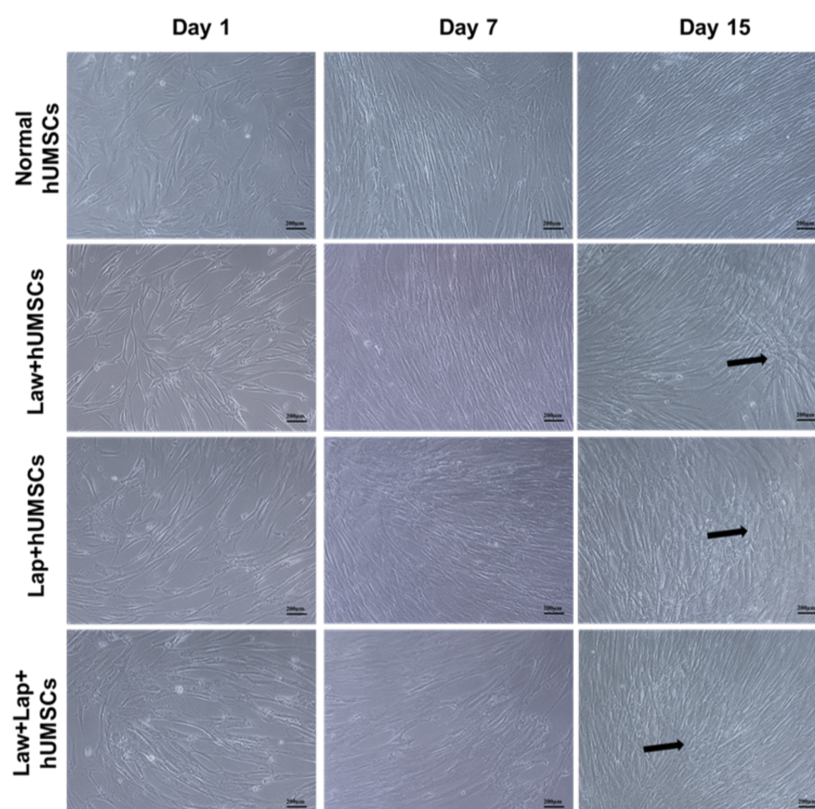

Fig. 2. hUMSC differentiation into IPCs *in vitro*: hUMSCs differentiated into insulin producing cells (IPCs) following their treatment with lawsone (Law), lapachol (Lap), or their combination (Law + Lap) after 15 days. Law + hUMSC group showed more clusters representing islet formation (black arrows) compared to other groups. All images were taken under phase contrast microscope.

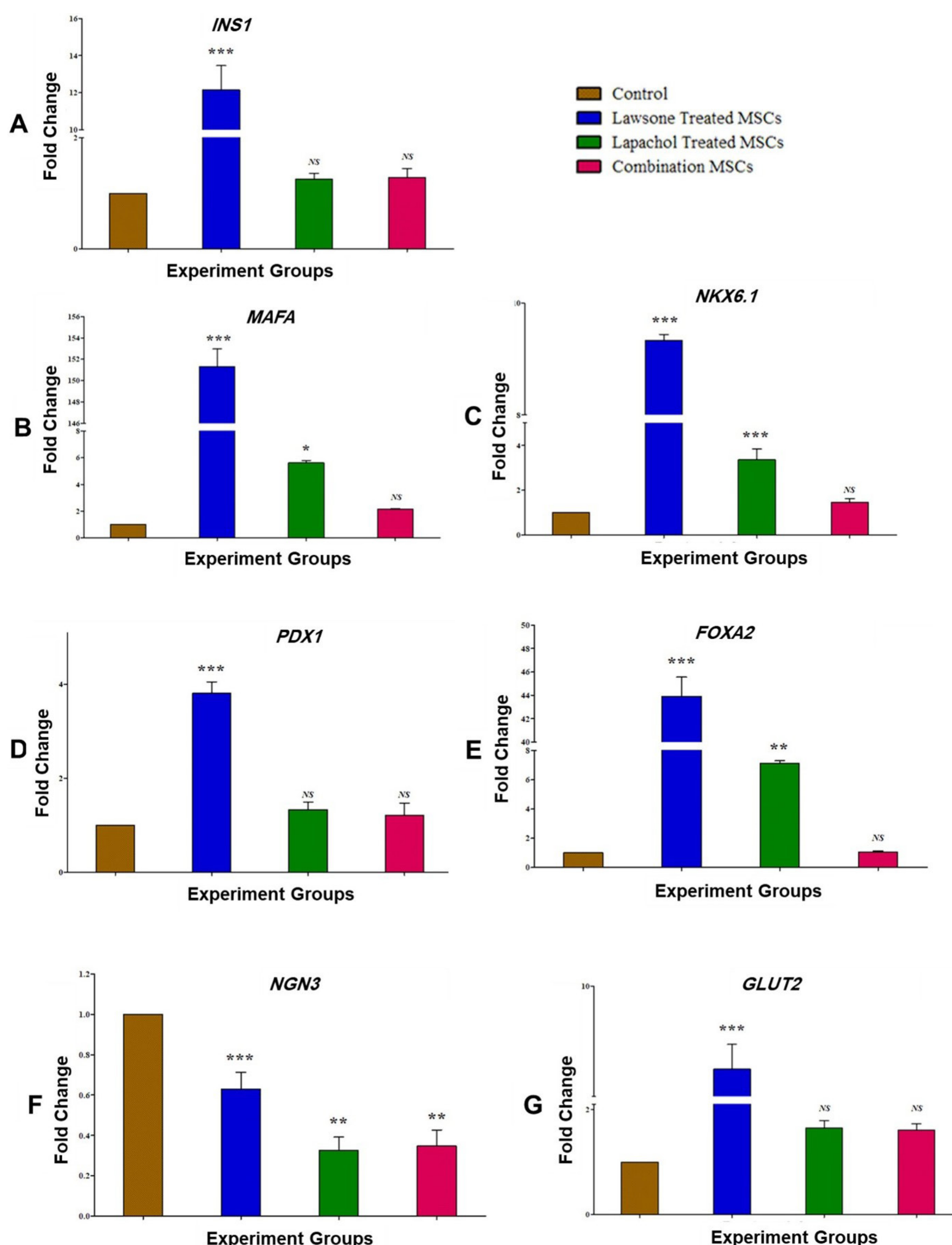

Fig. 3. Gene expression analysis of pancreatic β-cell markers: hUMSCs treated with lawsone (Law), lapachol (Lap), or their combination (Law + Lap) were analyzed for the gene expression of **(A)** *INS-1*, **(B)** *MAFA*, **(C)** *PDX1*, **(D)** *FOXA2*, **(E)** *NKX6.1*, **(F)** *GLUT2,* and **(G)** *NGN3*, at day 15. Statistical comparisons were performed by one-way ANOVA with a post hoc Bonferroni test. The statistical values are reported as mean ± SEM with n = 3 and a significance value of $P < 0.05$ (non-significant ^{NS}, $P < 0.05$*, $P < 0.01$**, and $P < 0.001$***).

In silico analysis

Molecular docking simulations showed that both lawsone and lapachol formed hydrogen bonds within the seven-transmembrane domain of the FZD receptor, with binding energies of -5.7 kJ/mol and -6.9 kJ/mol, respectively. Docking with the ALK-1 receptor indicated that these compounds formed hydrogen bonds within the serine/threonine kinase domain, with similar maximum binding energies of -5.7 kJ/mol for lawsone and -6.9 kJ/mol for lapachol. The cumulative atomic-level interactions yielded weighted binding scores of -7.2 for lawsone and -6.9 for lapachol (Fig. 7), showing their potential affinity for key components of the Wnt and BMP signaling cascades.

Discussion

Naphthoquinones and their structural analogs have been widely investigated for their potential therapeutic effects in diabetes treatments.[20,21] It is reported that naphthoquinone rich crude extracts of medicinal plants including *Lawsonia inermis*[22,23] and *Tabebuia*

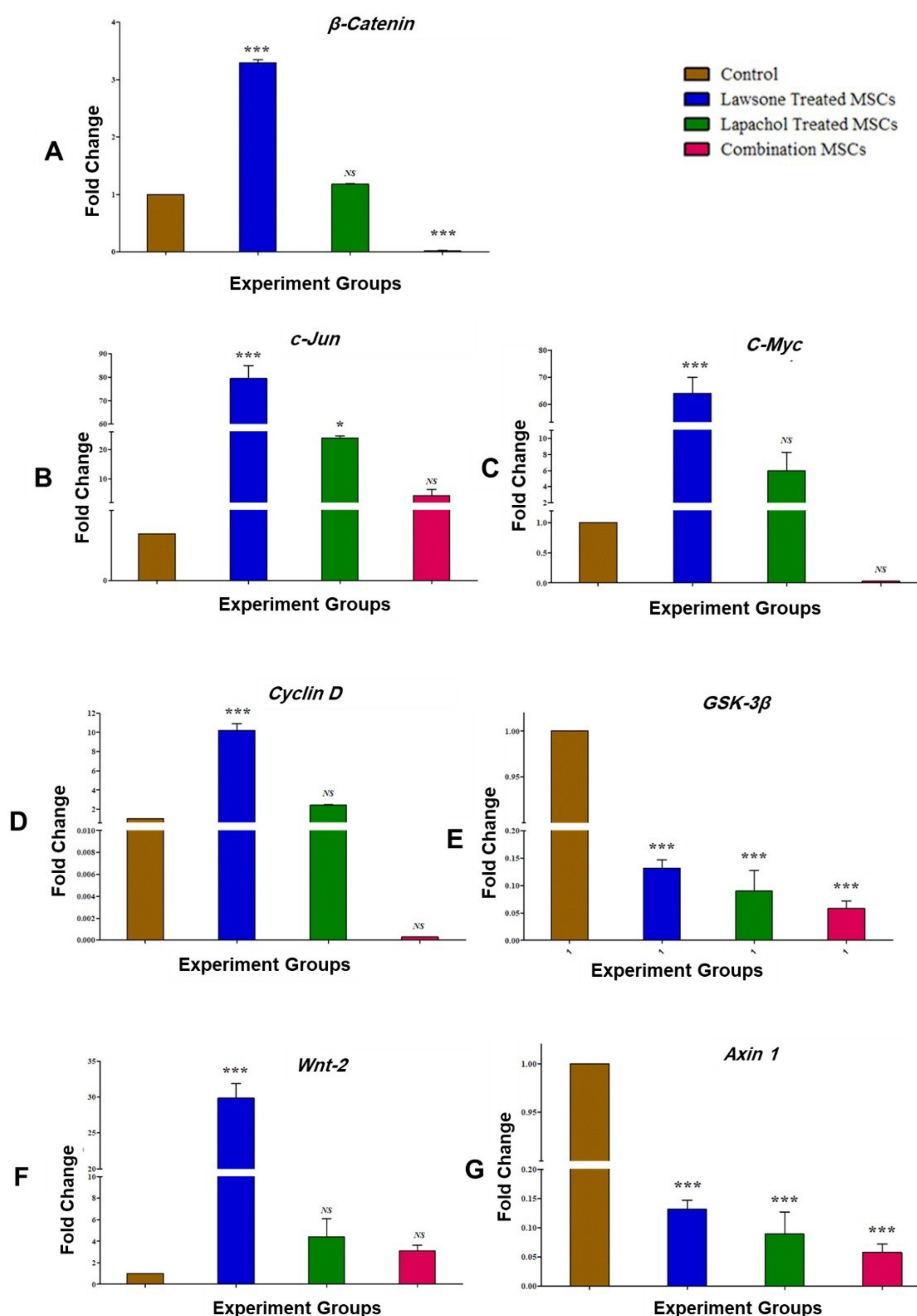

Fig. 4. Gene expression analysis of Wnt pathway mediators: hUMSCs treated with lawsone (Law), lapachol (Lap), or their combination (Law + Lap) were analyzed for the gene expression of **(A)** *Wnt-2*, **(B)** *c-Myc,* **(C)** *c-Jun*, **(D)** *Cyclin D*, **(E)** *β-Catenin*, **(F)** *GSK-3β*, and **(G)** *Axin1*, at day 15. Statistical comparisons were performed by one-way ANOVA with a post hoc Bonferroni test. The statistical values are reported as mean ± SEM with n = 3 and a significance value of $P < 0.05$ (non-significant ^{NS}, $P < 0.05$*, and $P < 0.001$***).

avellanedae[24,25] possess anti-diabetic activity.[26-29] Several studies have shown that MSCs can differentiate into IPCs *in vitro* using conditioned media, varied culture conditions,[30,31] and/or specific transcription factors.[32] Many investigators have evaluated the use of conditioned media in combination with the augmented mixture of non-essential amino acids, high-glucose, nicotinamide, activin A, and β-cellulin cocktail for the differentiation of IPCs as an alternate strategy for T1DM management.[33-35]

This study examined the role of naphthoquinones, lawsone and lapachol for their potential to differentiate hUMSCs into IPCs. The optimized concentrations of these compounds individually and in combination were used to treat hUMSCs for 2 weeks in serum free DMEM. Transcriptional analysis showed the overexpression of mature pancreatic β-cell markers *MAFA*, *INS-1*, *PDX1*, *FOXA2*, *GLUT2,* and *NKX6.1*, in all treated groups. However, lawsone treated group showed pronounced

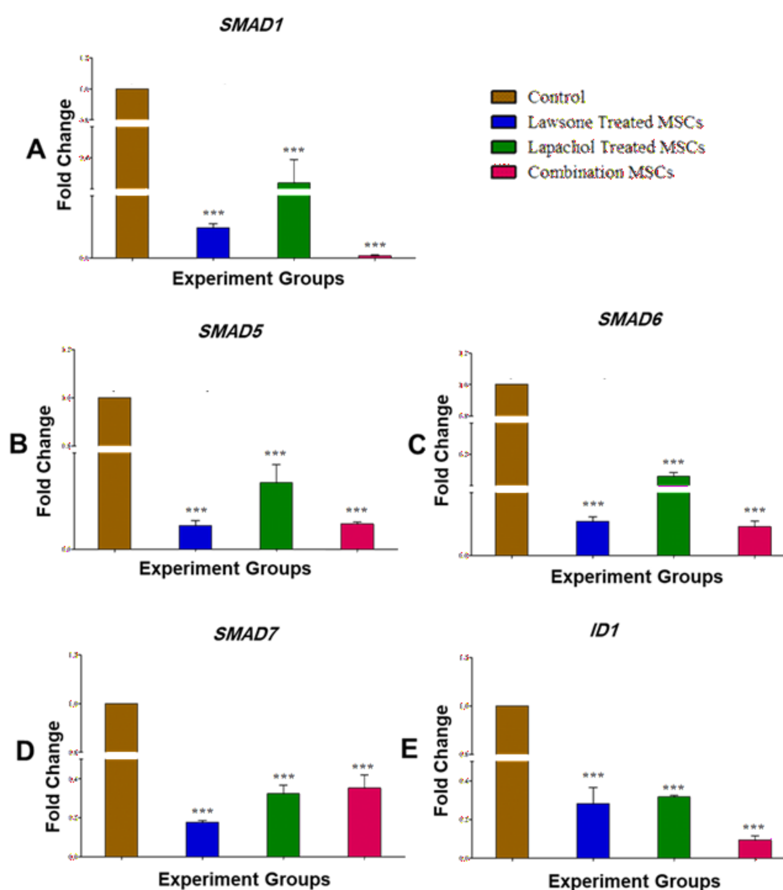

Fig. 5. Gene expression analysis of BMP pathway mediators: hUMSCs treated with lawsone (Law), lapachol (Lap), or their combination (Law + Lap) were analyzed for the gene expression of **(A)** *SMAD1*, **(B)** *SMAD5*, **(C)** *SMAD6*, **(D)** *SMAD7,* and **(E)** *ID1*, at day 15. Statistical comparisons were performed by one-way ANOVA with a post hoc Bonferroni test. The statistical values are reported as mean ± SEM with n = 3 and a significance value of $P < 0.05$ ($P < 0.001$***).

expression of these genes. Furthermore, *NGN3* expression was decreased in all groups.

The combined function of *MAFA*, *PDX1*, and *NGN3* is widely recognized in the pancreatic β-cell formation.[36,37] However, *NGN3* specifies the early-stage of pancreatic development and is absent in mature β-cells.[38] Conversely, *MAFA* and *PDX1* are recognized as key mediators of β-cell function and remain actively expressed in fully mature β-cells.[39] *FOXA2* is crucial not only for β-cell formation[40] but also for their maturation and the regulation of functional activity.[41] *NKX6.1* has a fundamental role in regulating the development, maturity, and proliferation of pancreatic β-cells.[42] The combination of both *PDX1*$^{+}$/*NKX6.1*$^{+}$and *PDX1*$^{-}$/*NKX6.1*$^{+}$genes has been found to effectively transform human pluripotent stem cells into IPCs.[43] *GLUT2* functions as a glucose transporter and is specifically found in fully developed β-cells.[44] According to some literature, *GLUT2* may not have a major effect on insulin secretion or glucose uptake.[45,46] However, other investigations indicate that their role in diabetes is greatly enhanced.[47,48]

We also evaluated the differentiation of IPCs *via* immunocytochemistry and our findings showed increased expression of insulin, a key protein produced by β-cells,[49] in all the treated groups, with the highest expression in the lawsone treated group. Additionally, glucagon, which is a protein exclusive to α-cells, showed minimal to no expression.

The main cause of T1DM is the dysfunction of pancreatic β-cells. These cells are highly regulated by a network of intrinsic and extrinsic signals that maintain their normal activity and promote proliferation in response to high insulin demands. Among various signaling pathways, Wnt pathway plays a vital role in the development and function of β-cells,[50-52] and its activation has been shown to enhance β-cell proliferation and functional maturation.[53-55] Our study also assessed the role of Wnt signaling for the commitment of hUMSCs into IPCs, under *in vitro* settings. Gene expression analysis revealed a significant downregulation of *GSK3β* and *Axin1* levels across all treatment groups. In comparison, the lawsone-treated group revealed overexpression of *Wnt-2, c-MYC, Cyclin D, c-JUN*, and *β-catenin* relative to the control and other treatment groups. These findings align with previous studies showing over-suppression of *GSK3β* expression. A study also reported higher expression of *β-catenin, Cyclin D*, and *c-MYC,* both *in vitro* and *in vivo* STZ-induced diabetic mice after gymnemic acid treatment.[56] Several other studies have also reported increased pancreatic β-cell proliferation when *GSK3β* is downregulated.[57,58] Similar observations have been shown that inhibition of GSK3β through pharmacological agents

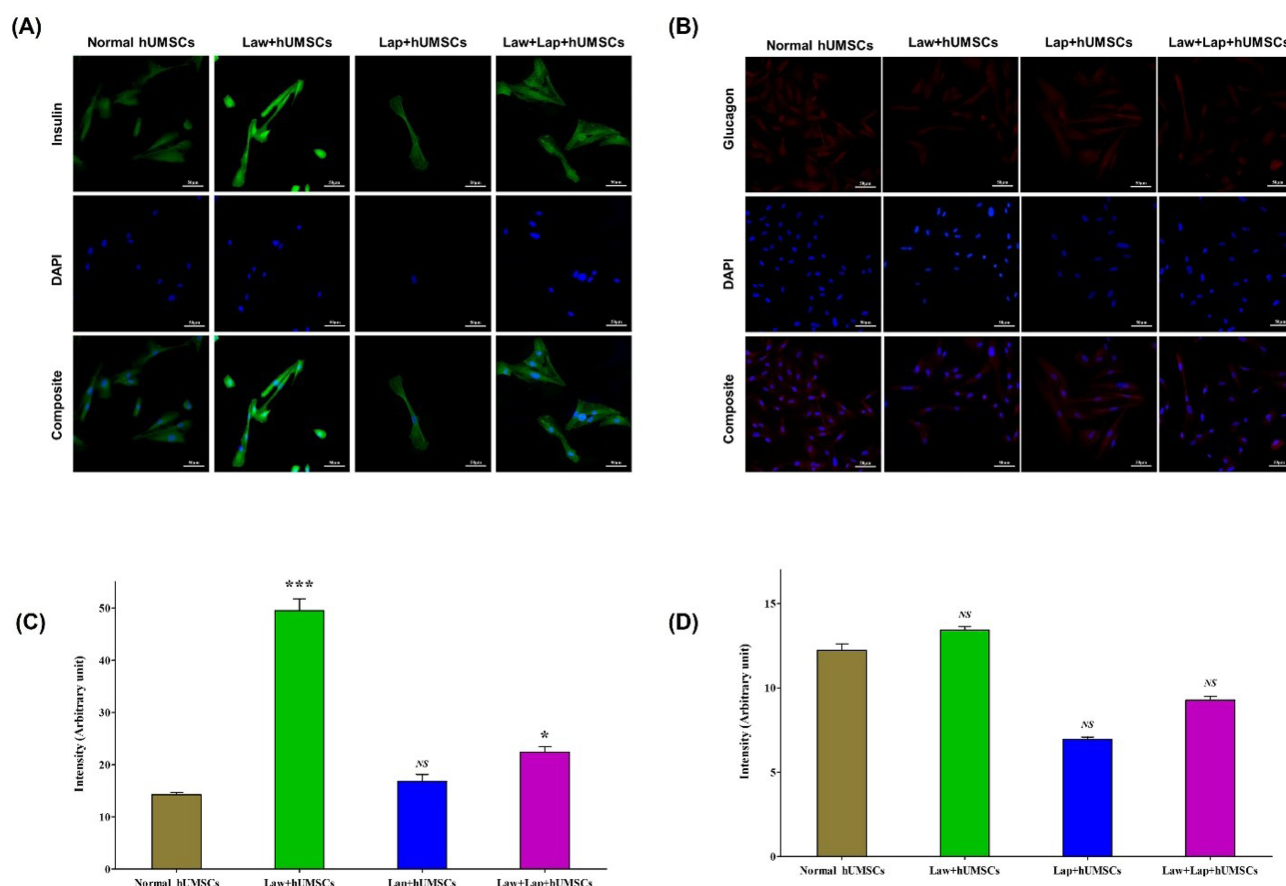

Fig. 6. Insulin and glucagon expression in treated hUMSCs: hUMSCs treated with lawsone (Law), lapachol (Lap), or their combination (Law + Lap), were analyzed for the protein expression of **(A)** insulin and **(B)** glucagon, compared to the control group. Images were taken under fluorescent microscope. **(C)** Quantitative analysis showed fluorescent intensities of insulin indicating positive expression. **(D)** All the treated groups showed negative expression of glucagon, an α-cell specific marker. Statistical comparisons were performed by one-way ANOVA with a post-hoc Bonferroni test. The statistical values are reported as mean ± SEM with n = 3 and a significance value of $P < 0.05$ (non-significant ^{NS}, $P < 0.05$*, and $P < 0.001$***).

or genetic modification promoted β-cell survival and expansion through stabilization of β-catenin.[59-61]

Numerous studies suggest that inhibition of SMAD proteins[62] and ID1[63] increase pancreatic β-cell proliferation, indicating that their overexpression is involved in the development of diabetes.[64,65] Findings from our study showed inhibition of *SMADs* and *ID1* at the transcriptional level, corresponding to the suppression of the BMP pathway, thereby promoting pancreatic β-cell proliferation. Previous studies have also demonstrated that stage-specific inhibition of BMP signaling enhances the efficiency of stem cell differentiation into insulin-producing cells supporting our observations.[66-68]

We have also analyzed the interaction of lawsone and lapachol with Wnt and BMP receptors via *in silico* analysis. Blind docking demonstrated the binding of the ligands i.e., lawsone or lapachol to the seven-transmembrane domain of FZL at the N-terminal region. Numerous studies have demonstrated that Wnt2 has a strong affinity for the N-terminus of FZL; therefore, their binding leads to the activation of the downstream signaling cascade through the Wnt canonical pathway. It has been shown that there was a substantial reduction in β-cell mass in rats when cells were exposed to TCF7L2, a canonical Wnt pathway inhibitor. This demonstrates the significance of the canonical Wnt pathway in paracrine functions.[69-72] Our study revealed that Law + FZL and Lap + FZL had comparable amino acid sequences, both involved in the binding of the Wnt2 ligand to the FZL receptor.[73]

Lawsone and lapachol have been reported to bind to the serine/threonine kinase domain of the BMP pathway receptor, ALK-1. Type 1 kinases, upon activation through the same domain, phosphorylate the SMAD proteins.[74,75] Previous studies have shown that inhibiting BMP type I receptors can reduce BMP-induced phosphorylation of SMAD1/5/8, resulting in differentiation of stem cells.[76] Small-molecule inhibitors such as K02288 have been reported to effectively block the kinase activity of ALK receptors, leading to suppression of downstream SMAD-mediated transcriptional signaling.[77] Regulation of the BMP pathways is therefore considered an important mechanism in stem cell fate decisions, since it can modulate signaling pathways that maintain progenitor characteristics and control lineage differentiation.[78] Interestingly, our investigation demonstrated that the Law + ALK-1 and Lap + ALK-1 complexes involve amino acid residues similar to those reported for the K02288 + ALK-1 interaction,[77] suggesting a comparable inhibitory mechanism that may contribute to the differentiation of hUMSCs into IPCs.

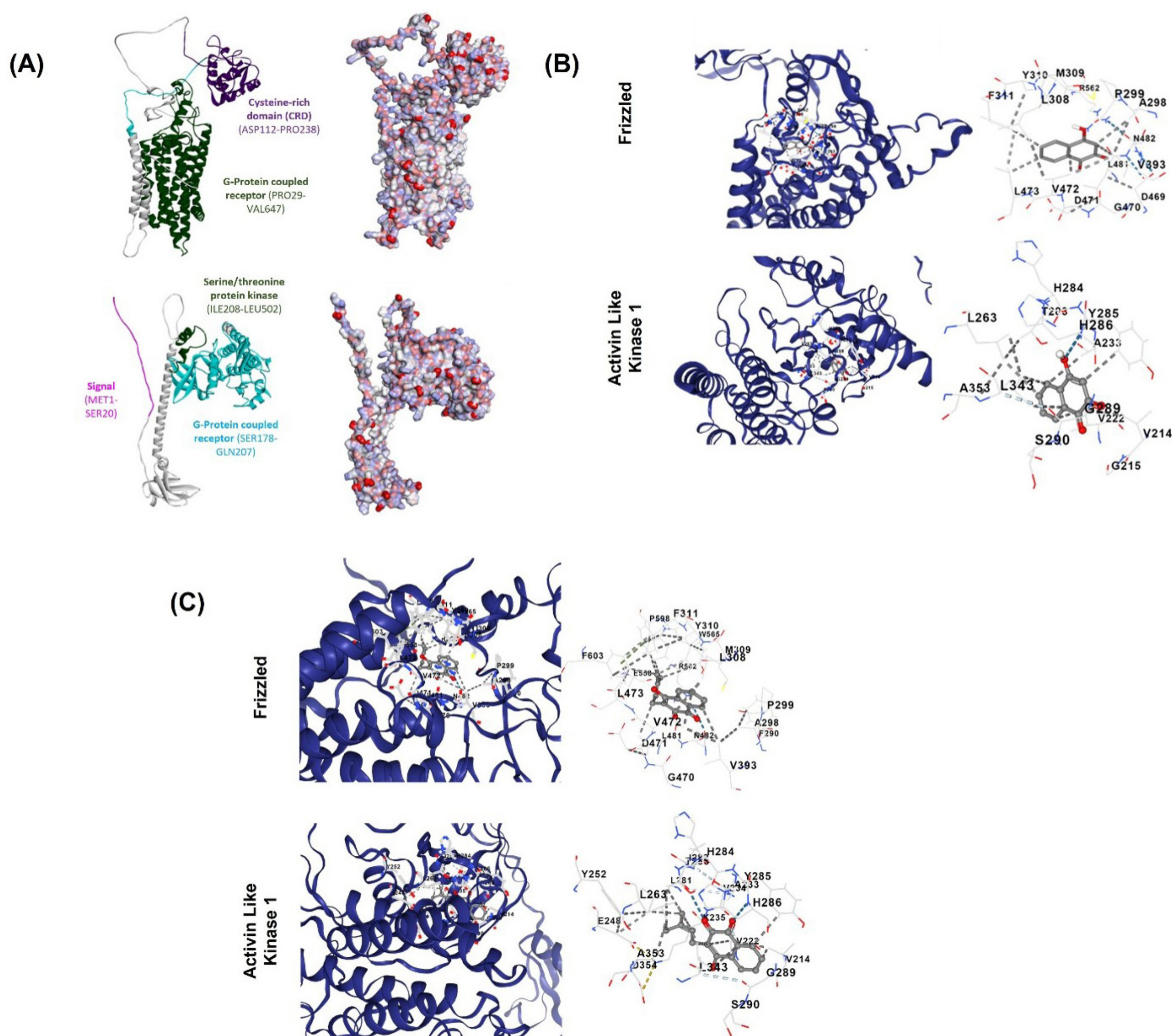

Fig. 7. *In silico* analysis of lawsone and lapachol with FZD and ALK1. (A) Three-dimensional ribbon structure and electrostatic surface topology of FZD (top) and ALK1 (bottom) receptors. The active domains present in the receptors are also highlighted. (B) Binding patterns reveal that both ligands occupy well-defined pockets within the receptor cavities, stabilized by a network of hydrogen bonds and hydrophobic interactions. Lawsone exhibits moderate binding affinity (-5.7 kcal/mol), forming key polar contacts with active site residues in FZD and ALK1. (C) Lapachol demonstrates stronger binding affinity (-6.9 kcal/mol), characterized by hydrophobic interactions and additional stabilizing contacts within the receptor pocket, suggesting improved binding stability. The interaction plots further describe the specific amino acid residues involved in ligand coordination, emphasizing critical hydrogen bonding and nonpolar interactions that direct ligand-receptor complex formation. All docking simulations were performed using CB-Dock under standard parameters with a blind docking approach.

Limitations of the study

Functional validation of insulin-producing cells including glucose-stimulated insulin secretion, C-peptide release, and insulin content analyses, is required to support our findings. The long-term stability, maturation, and functional competence of the differentiated β-like cells should also be established and will be evaluated in future studies.

Conclusion

The current study demonstrated the role of naphthoquinones *i.e.*, lawsone and lapachol, in the differentiation of hUMSCs into IPCs. Among all groups analyzed, lawsone treated group showed significant overexpression of β-cell specific markers, showing its potential for hUMSC commitment toward IPCs both at gene and protein levels. Although, lapachol treated hUMSCs showed reduced expression of β-cell markers as compared to the lawsone treated cells, their expression was still higher in comparison to the untreated control. Optimization of culture conditions is required to enhance the potential of lapachol for efficient differentiation of hUMSCs. Additionally, the study has reported the potential of naphthoquinones as a potent regulator of Wnt and BMP signaling pathways which was evident by the modulation of the specific key molecules at the gene level. *In silico* analyses also showed similar binding patterns of lawsone or lapachol with respective receptors of Wnt and BMP pathways. Therefore, regulatory role of these compounds can be exploited in the pancreatic β-cell regenerative applications for the management of T1D.

Acknowledgements
This project is financially supported by PhD Indigenous Fellowship provided by Higher Education Commission, Pakistan to Dr. Javeria Masnoon. We are also thankful to Zainab Panjwani Memorial Hospital, Karachi, for providing us the umbilical cord tissue samples.

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Competing Interests
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon request.

Declaration of AI-assisted Tools in the Writing Procedure
None.

Ethical Approval
The study approved by the Institutional Ethics Committee with the assigned protocol number, "ICCBS/IEC-037-HT-2018/Protocol/1.0". Cord tissue samples were obtained from healthy donors undergoing full-term cesarean deliveries at the Zainab Panjwani Memorial Hospital, Karachi, after obtaining their written informed consent.

Funding
This project is financially supported by PhD Indigenous Fellowship provided by Higher Education Commission, Pakistan to Dr. Javeria Masnoon.

Supplementary files
Supplementary file 1 contains Table S1.

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