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Computational Analysis of miR-140 and miR-135 as Potential Targets to Develop Combinatorial Therapeutics for Degenerative Tendinopathy

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Background: Degenerative tendinopathy, a condition causing movement restriction due to high pain, highly impacts productivity and quality of life. The healing process is a complex phenomenon and involves a series of intra-cellular and inter-cellular processes. Proliferation and differentiation of the tenocyte is a major and essential process to heal degenerative tendinopathy. The recent development in microRNA (miRNA)-mediated reprogramming of the cellular function through specific pathways opened door for the development of new regenerative therapeutics. Based on information about gene expression and regulation of tendon injury and healing, we attempted to evaluate the combinatorial effect of selected miRNAs for better healing of degenerative tendinopathy.

Methods: The present study was designed to evaluate the combinatorial effect of two miRNAs (has-miR-140 and has-miR-135) in the healing process of the tendon. Publicly available information/data were retrieved from appropriate platforms such as PubMed. Only molecular data, directly associated with tendinopathies, including genes/proteins and miRNAs, were used in this study. The miRNAs involved in tendinopathy were analyzed by a Bioinformatics tools (e.g., TargetScan, miRDB, and the RNA22v2). Interactive involvement of the miRNAs with key proteins involved in tendinopathy was predicted by the Insilco approach.

Results: Based on information available in the public domain, tendon healing-associated miRNAs were predicted to explore their therapeutic potentials. Based on computation analysis, focusing on the potential regulatory effect on tendon healing, the miR-135 and miR-140 were selected for this study. These miRNAs were found as key players in tendon healing through Rho-associated coiled-coil containing protein kinase 1 (ROCK1), IGF-1/PI3K/Akt, PIN, and Wnt signaling pathways. It was also predicted that these miRNAs may reprogram the cells to induce proliferation and differentiation activity. Many miRNAs are likely to regulate genes important for the tendinopathy healing process, and the result of this study allows an approach for miRNA-mediated regeneration of the tenocyte for tendon healing. Based on computational analysis, the role of these miRNAs in different pathways was established, and the results provided insights into the combinatorial approach of miRNA-mediated cell reprogramming.

Conclusions: In this study, the association between miRNAs and the disease was evaluated to correlate the tendinopathy genes and the relevant role of different miRNAs in their regulation. Through this study, it was established that the synergistic effect of more than one miRNA on directed reprogramming of the cell could be helpful in the regeneration of damaged tissue. It is anticipated that this study will be helpful for the design of miRNA cocktails for the orchestration of cellular reprogramming events.

Keywords: *Tendinopathy, Regenerative medicine, MicroRNA, Therapeutics, Extracellular matrix remodeling*

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Degenerative tendinopathy is a major challenge in this aging world. Sustaining pain and functional impairment due to degeneration in the tendon highly obstruct the daily productivity and capacity to work. For this degenerative condition of the tendon, various factors are responsible.[1-3] Tendon is one of the strongest connective tissues, which helps in managing strain and maintaining stress balance between muscle and bone. The physiological load causes more than 4% expansion in the tendon, resulting in the rupture of one or more fiber bundles in tendon tissue. The physical load, which may expand the tendon by more than 8% may cause a complete rupture; however, in some cases, some individuals can tolerate a load resulting in a 12% tendon expansion.[4] The heavy physical load is not always a culprit for inducing tendinopathy, a repetitive microtrauma due to overuse of this tissue may also cause the disease.[5] Medically, two theories are highly acceptable on tendon injury: (1) mechanical and (2) vascular theory. However, these two theories are not mutually exclusive.[6] In general, acute injuries are the result of disruption/rupture of the tendon matrix, which can be slowly repaired by the resident cells. However, in degenerative/chronic tendon diseases (tendinopathy), depletion in resident cell counts and vital activities are common phenomena. As mentioned above, the degenerative tendon condition is a result of the collective involvement of anomalies in various biochemical pathways. Therefore, its healing is also complex and depends on various factors including the individual's health and lifestyle.[7] The healing of the tendon involves consecutive steps of hemostasis, proliferation, and remodeling (Fig. 1). There are reports on the interaction between important genes and various factors (intrinsic and extrinsic) that can influence the healing processes. It has been identified that more than 280 genes are significantly involved in tendinopathy.[8] Tenocyte proliferation is an important step in the intrinsic healing process; therefore, the recruitment of resident cells is an important step, which is required for matrix remodeling and proper healing of the tendon.[5,6] Matrix remodeling and achieving homeostasis are a very complex phenomenon that includes gene expression routing and epigenetically regulated signaling and pathway activation. Among various factors, the noncoding (RNAs), such as small interfering RNAs (siRNAs), and microRNA (miRNA), have been identified as key regulators for the various biological pathways. In the past two decades, scientists have successfully identified several miRNAs, which are involved in the development of various diseases and also in the healing process.[9-11] As mentioned above, the potential of miRNA in the post-transcriptional regulation of gene expression can be applied for target identification as well as therapeutics development. Biologically the miRNAs usually bind to 3'-untranslated region (UTR) of the mRNAs, which results in negative regulation of gene expression either by inhibiting protein translation or by mRNA degradation.[12] This up to 22-mer noncoding RNA, better known as miRNA, is transcribed from DNA, which has been seen as an explanation for some of the differential expression seen in mRNA splice variants prevalent in tendinopathy conditions.[13] The miRNAs are not only involved in downregulation, but also induce gene expression through binding to complementary regions in the promoter and the 5'-UTR.[14] It has been reported that miRNAs can control 90% of human transcripts.[15] There are many reports on multiple miRNA-mediated single gene regulation, as well as single miRNA-mediated multiple gene regulation. Therefore, they are probably involved in several biological activities including cell proliferation, differentiation, apoptosis, and matrix turnover.[1,3,16]

The omics-based approach used in this study is being used by several studies in discovering the number of biomarkers and therapeutics targets for various diseases.[17] Recently, the miRNAs have received huge attention from researchers and pharmaceutical companies. In an estimate, more than 33,500 articles containing a keyword of miRNAs have been archived in PubMed for the year 2021, while in the last 5 years, 123,930 articles were included in PubMed with an miRNA keyword. This shows the potential of this small non-coding RNA for controlling the molecular pathway to achieve homeostasis. In this study, a screening of the miRNA for its role in protein regulation

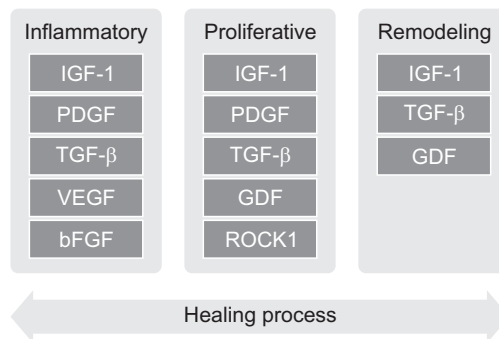

Fig. 1. A proposed process of tendon healing based on previous studies. The three consecutive steps, namely, inflammatory, proliferative, and remodeling, were identified and showed key regulatory proteins of the respective process. IGF-1: insulin-like growth factor 1, PDGF: platelet-derived growth factor, TGF-β: transforming growth factor beta, VEGF: vascular endothelial growth factor, bFGF: basic fibroblast growth factor, GDF: growth differentiation factor, ROCK1: Rho-associated coiled-coil containing protein kinase 1.

was evaluated.[18] The omics of tendon biology is at a very basic level; however, as much as information is revealed by the research, the omics of the tendon will also be enriched. Therefore, this study was designed to find an miRNA-mediated cell-free regenerative therapy solution for tendinopathy therapy. In a bioinformatics-based analysis, the hypothesis design is a crucial step. Therefore, careful selection of experimental groups, targets, proteins, miRNA, and genes is important to achieve an appropriate result. The computational analysis of miRNA and pathway analysis for the development of a new biomarker for diagnostics as well as the target for therapeutics development has been validated by the wet lab.[17,19,20] This study mainly focused on the role of miRNA combinations in tendon healing and also on identifying the related proteins involved in tendinopathy. This information may be helpful for the development of new therapeutics that can cure degenerative tendinopathy in the cell-free treatment process.

METHODS

The schematic diagram of the methodology used in this study is illustrated in Fig. 2. Before execution of the methodology, the study was evaluated by the professors at the Department of Orthopaedics, Kangnam Sacred Heart Hospital, Hallym University School of Medicine, Seoul, Korea and approved by the Institutional Review Board (No. 2021-11-033-002).

Data Collection and Literature Search on Tendinopathy and Related miRNA

The online information, previously published research articles, and archived data were used for this study. In this study, the screened data, which were found to be directly associated with tendinopathies such as genes/proteins and related miRNAs were sorted and presented in Table 1. Publicly available data were retrieved from an appropriate platform such as PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and the search criteria were limited to human studies. Manuscripts written other than in English, studies carried out on animals and cell lines, review articles, and experimentally non-validated data were not considered for this study. The keywords used for the search of articles was "Tendinopathy+miRNA." Different registries related to microarray databases on miRNA expression were surveyed from NCBI Gene Expression Omnibus (GEO) and other databases to refine the study and obtain enriched information.

Defining Tendinopathy-Related Genes

The set of miRNAs involved in tendinopathy was defined using available information in the database. The genes involved in tendon biology and the healing process were mined from the Pubmed and GEO databases.[21] The shortlisting of the potential target genes was based on known function, expression profile in tendinopathy, or involvement in tendon healing. Lists of genes involved in tendon biology were analyzed by Targetscan, miRDB, and the RNA22v2. Based on the database score, the genes were shortlisted for further study.[11]

miRNA Target Prediction

The miRNAs were predicted (Supplementary Fig. 1) by the TargetScan (an open-source database), and the miRNAs targeting the genes involved in tendon healing were considered for the present study.[22]

Construction of a Protein-Protein Interaction Network

The STRING database was used to construct the protein-protein interaction (PPI) under the standard protocol.[23] The STRING database provides enriched information about the PPIs on both the known and predicted. The selected genes were submitted to the database for network generation. All the analysis was done with human-centric information. However, the biological process related to tendinopathy was considered for further analysis.

Analysis of the PPI Network

The NetworkAnalyst tool[24] was used to create the PPI network. This tool is a web-based open-source analytics platform for the system-level interpretation of gene expression profiles. It also provides biomolecular interaction data, tissue-specific gene network analysis, and gene regulatory network, interpreting the biological process. To construct the PPI network, Homo sapiens (human) was selected as the organism, and another input such as the identification (ID) type and proteins was kept as the default setting. Among all available options, the "generic PPI" was selected

Fig. 2. Workflow of the study, showing methodology used in this study to shortlist the proteins and corresponding microRNAs (miRNAs). KEGG: Kyoto Encyclopedia of Genes and Genomes.

Table 1. Biological Function of the Reported miRNA and Major Pathway Involvement during Tendon Healing

Biological function	miRNA	Target	Study
Linked to inflammation	miR-146a-5p	JAK2/STAT3	Thankam et al. (2018)[10]
	miR-193b-3p		
	miR-195-5p	AMPK and TREM-1	Thankam et al. (2019)[25]
	miR-31-5p		
Cell proliferation and differentiation	miRNA-499	CUGBP2, MYB	Cai et al. (2015)[26]
	miR28-5p	p53 Deacetylase sirtuin 3	Poulsen et al. (2014)[27]
	mir-135		
	mir-140		
Homeostasis and remodeling	mir-140	PIN1	Chen et al. (2015)[28] Chen et al. (2015)[29]
	mir-135	ROCK1	Watts et al. (2017)[30] Ge et al. (2018)[31]
	miR-21-3p	p65	Yao et al. (2020)[32]
	miR-125a-5p		
	miR-145-5p	COL1A2, COL3A1,	Thankam et al. (2016)[33]
	miR-29a	MMP2 and MMP9	
	miR-151a-3p		
	miR-199a-5p		
	miR-382-5p		
	miR-498	IL-33/SST2	
	miR-148a-3p	KLF6	Millar et al. (2015)[34], Millar et al. (2021)[35]

miRNA: microRNA, JAK2: Janus kinase 2, STAT3: signal transducer and activator of transcription 3, AMPK: AMP-activated protein kinase, TREM-1: triggering receptor expressed on myeloid cells 1, CUGBP2: CUG triplet repeat, RNA binding protein 2, MYB: transcriptional activator Myb, PIN 1: peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, ROCK1: Rho-associated coiled-coil containing protein kinase 1, p65: nuclear factor NF-Kappa-B P65 subunit, COL1A2: collagen type I alpha 2 chain, COL3A1: collagen type III alpha 1 chain, MMP2: matrix metallopeptidase 2, MMP9: matrix metallopeptidase 9, IL33: interleukin 33, SST2: somatostatin 2, KLF6: Krueppel-like factor 6.

Table 2. List of miRNA Reported in the Literature in Association with Tendinopathy

Up	Down	Study
miR-7a, miR-5p	let-7g-5p, miR-135-3p, miR-135-5p, miR-140-3p, miR-18b-5p, miR-19a-3p, miR-19b-3p, miR-192-5p, miR-210-3p, miR-22-3p, miR-222-3p, miR-25-3p, miR-26a/b-5p, miR-29a-3p miR-29a/b, miR-29a-3p, miR-30a-5p, miR-324-3p, miR-378a, miR-425-5p, miR-29c-3p, miR-30a-5p, miR-192-5p, miR-93-5p,	Thankam et al. (2018)[10], Lu et al. (2017)[36], Liu et al. (2019)[37], Han et al. (2017)[38], Han et al. (2021)[39], Plachel et al. (2020)[9], Chen et al. (2015)[28], Chen et al. (2015)[29], Wang et al. (2016)[40], Ding et al. (2021)[41], Zhu et al. (2021)[11]

miRNA: microRNA.

for further analysis (Supplementary Fig. 2). The threshold value for the confidence score was set at 0.900 for the PPI study.[42]

Kyoto Encyclopedia of Genes and Genomes Pathway and Gene Ontology Analysis

The DAVID database was used for gene ontology (GO) functional annotation and validation of the predicted cor-

relation.[43] It also analyzed the molecular function, the biological process, and cellular component during the study. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and DAVID were available as open-source platforms and allowed a wide range of studies. Using the above tool, the prediction of miRNA and their involvement in tendinopathy was performed.

Constructing a miRNA-Gene Regulatory Network

To establish a correlation between the miRNAs and genes involving tendinopathy, the miRNA-gene network was constructed (based on previously reported genes) (Table 2).[24] To perform this study, the miRNet (an open-source tool for the statistical analysis of miRNA data) was used.[44] Interaction between the miRNA and transcription factor was predicted by the TRRUST (Transcriptional Regulatory Relationships Unraveled by the Distance-based Text-mining) database.[45]

RESULTS

Prediction of miRNA

Tendinopathy is a degenerative medical condition where the tendon fails to heal on its own. It can easily be characterized by a chaotic proliferation of tenocytes, an imbalanced synthesis of collagen and other matrix components, and a high level of inflammatory molecules.[46] Tendon healing has three important steps: i.e., response against

Table 3. Ontology of the Predicted miRNA and Their Context Score

Gene targeted	miRNA involved	Biological function
ROCK1	miR-135-5p, miR-140-3p, miR-101-3p, miR-15-5p, miR124-3p, miR-129-5p miR-132-3p, miR-139-5p, miR-142-5p, miR-144-3p, miR-145-5p, miR-150-5p, miR-153-3p, miR-182-5p, miR- 190-5p, miR-199-5p, miR-217, miR-212-3p, miR-214-5p, miR-26-5p, miR-302c-3p, miR-31-5p, miR-33-5p, miR-34-5p, miR-148-3p, miR-152-3p, miR-129-5p, miR-449-5p,	ROCK1 is an important regulator of actin-myosin contraction and cell polarity; therefore, it is involved in various biological functions. They have a significant role in healing and remodeling of ECM as their direct involvement in the regulation of cell proliferation, differentiation, morphology, apoptosis/senescence.
PDGF	miR-135-5a, miR-1-3p, miR-221-3p, miR-222-3p, miR-153-3p, miR-124-3p, miR-506-3, miR-194-5p, miR-23-3p, miR-375, miR-29-3p, let-7-5p, miR-17-5p, miR-20-5, miR-93-5p, miR-106-5p, miR-519-3p	PDGFs are reported as wound healing promoting a molecule, which also regulates the blood vessel tonus, and maintaining the homeostasis of the interstitial fluid pressure.
IGF-1	miR-135p, miR-29-3p, let-7-5p, miR-98-5p, miR221-3p, miR-222-3p, miR-142-5p, miR-148-3p, miR-152-3p, miR-425-5p, miR-15-5p, miR-16-5p, miR-195-5p, miR-424-5p, miR-497-5p, miR-27-3p, miR-199-3p, miR-26-5p, miR-29-3p, miR-130-3p, miR-301-3p, miR-454-3p, miR-196-5p, miR-128-3p, miR-19-3p, miR-18-5p, miR128-3p, miR-190-5p, miR-130-3p, miR-301-3p, miR-454-3p, miR-19-3p, miR-9-5p, miR-338-3p	IGF-1 plays a pivotal role in fetal development, adolescent growth, and adult tissue homeostasis. Together with insulin and growth hormone, IGFs regulate glucose and lipid metabolism, and thereby regulate body composition.
VEGF	miR-140-5p, miR-140-3p, miR-205-5p, miR-15-5p, miR-16-5p, miR-195-5p, miR-242-5p, miR-29-3p, miR-200bc-3p, miR-1-3p, miR-383-5p, miR-199-5p, miR-302-3p, miR-372-3p, miR-520-3p, miR-17-5p, miR-20-5p, miR-93-5p, miR-106-519-3p	VEGF is involved in vasculogenesis, angiogenesis, and lymphangiogenesis during embryonic and postnatal development.
TGF-β	miR-140, miR-135-3p, miR-200a-3p, miR-133a-3p, miR-133b-3p, miR-193-3p, miR-145-5p, miR-148-3p, miR-152-3p, miR-203a-3p, miR-301-3p, miR-199-5p, miR-29-3p, miR-142-5p, miR-21-5p, miR-153-3p, miR-203-3p, miR-145-3p, miR-141-3p, miR-23-3p, miR-25-3p, miR-32-5p, miR-92-3p, miR-363-3p, miR-367-3p	TGF-β superfamily is well cited for their active role in tendon healing. They are the key player for the cell growth, proliferation, differentiation, and ECM remodeling, and collagen metabolism.
bFGF	miR-135, miR-15-5p, miR-16-5p, miR-103-3p, miR-148-3p, miR-152-3p, miR-155-3p, miR-199-3p, miR-190-5p, miR-499a-5p, miR-101-3p, miR-15-5p, miR-16-5p, miR-195-5p, miR-424-5p, miR-497-5p, miR-203a-3p, miR-129-5p, miR-202-5p, miR-214-5p	FGF has diverse effects in a different cell. FGF-2 is a strong stimulant for angiogenesis. Therefore, it plays a significant role in tissue repair and wound healing. FGF-2 may also stimulate the differentiation process in the musculoskeletal system.
GDF	miR-140, miR-296-5p, miR-3147, miR-4425, miR-5701, miR-325-3p, miR-3186-3p, miR-4468, miR-6081	It is involved in the regulation of inflammatory pathways. It is a growth and differentiation factor directly associated with the regulation of cell growth, cell repair, and senescence, which are important for tendon healing.

miRNA: microRNA, ROCK1: Rho-associated coiled-coil containing protein kinase 1, ECM: extracellular matrix, PDGF: platelet-derived growth factor, IGF-1: insulin-like growth factor 1, VEGF: vascular endothelial growth factor, TGF-β: transforming growth factor beta, bFGF: basic fibroblast growth factor, GDF: growth differentiation factor.

inflammation, cell proliferation or mitotic and differentiation process, and matrix remodeling by collagen and peptidoglycan homeostasis (Fig. 1). The miRNAs against key regulatory genes were predicted by Targetscan (https://www.targetscan.org/vert_72/). TargetScan allows for predicting miRNA against specific genes by identifying the conserved sites that complement the miRNA's seed region.[22,47] The seed region of the miRNA is the sequence, which binds to the complementary sequences of the target mRNA. It also identifies the sites with mismatches in the centered sites and seed region.[47] The prediction ranking was based on the cumulative weighted context++ scores.[48] The context++ is a model developed by Agarwal et al.,[48] which uses 14 features (such as site type, supplementary pairing, local adenine uracil contents, minimum distance, and open reading frame of the gene length); therefore, it is more predictive than another model. TargetScan predictions incorporate both the context++ scores and current isoform information when ranking mRNAs with canonical 7–8 nt miRNA sites in their 3'-UTRs. The miRNA predicted against the genes that play an important role in tendon healing (Table 3). The binding region of each miRNA was provided in Supplementary Fig. 1. This insight was important for selecting miRNA for further study.

Protein and Pathway Identification

Restoration of the inherent structure of the tendon is the main goal of tendon regeneration to ensure its biological functions.[49,50] Involvement of the selected miRNA in protein expression related to tendon headlining or tenogenesis is provided in Table 3. It is well known that growth factors have a potential role in cell proliferation and matrix synthesis.[51-53] Change in the expression profile of the cytokines and growth factors in response to injury and healing is a key phenomenon. Tendon healing may broadly be divided into three steps: (1) inflammatory, (2) proliferative, and (3) extracellular matrix (ECM) remodeling. Among different key proteins, insulin-like growth factor 1 (IGF-

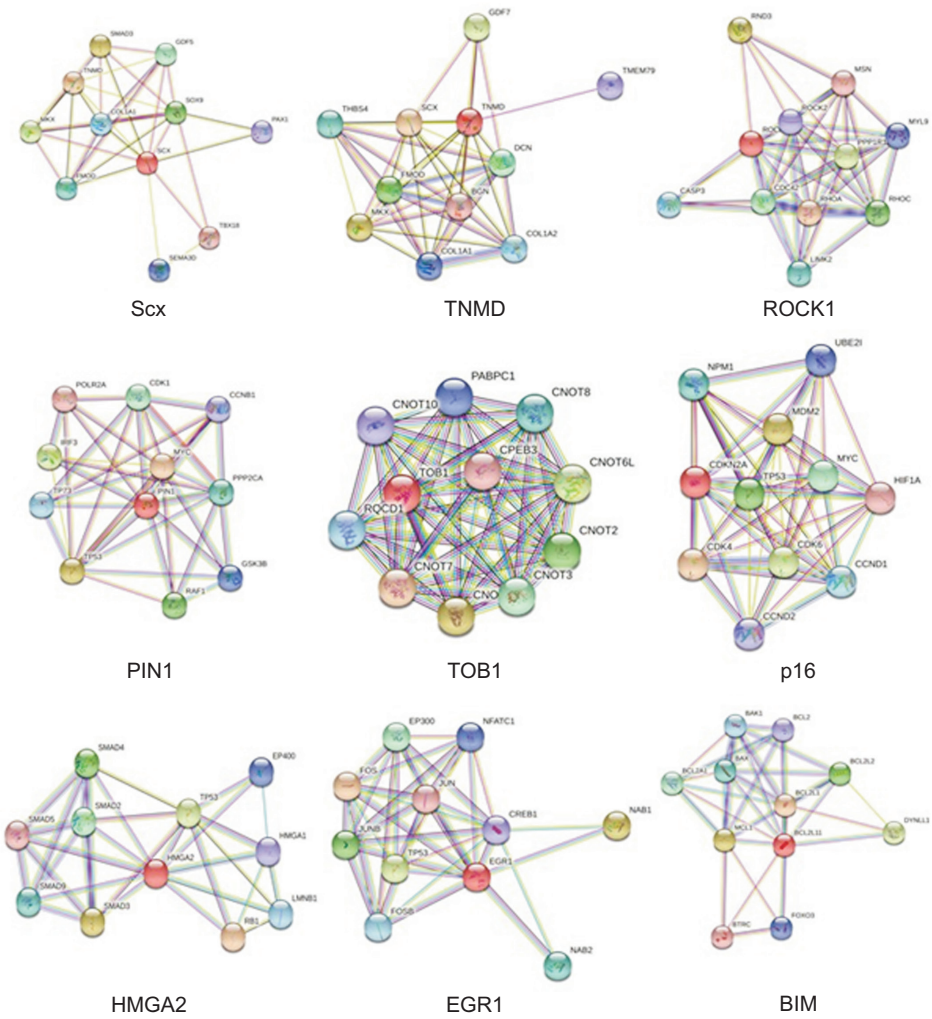

Fig. 3. The key proteins, their involvement in tendinopathy, and their interaction with other proteins. These proteins collectively responsible for tendon healing were used for protein-protein interaction network analysis to predict the involvement of the major pathway. ROCK1: rho-associated coiled-coil protein kinase 1, PIN1: peptidyl-prolyl cis/trans isomerase NIMA-interacting 1, TOB1: transducer of ERBB2, 1, HMGA2: high-mobility AT-hook 2, EGR1: early growth response-1.

1) and transforming growth factor beta (TGF-β) have a significant role in all three stages of healing.[53] Changes in the expression profile of TGF-β, IGF-1, interleukin (IL)-6, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) were reported during the initial phase of inflammatory responses (Fig. 1).[54] However, in the proliferation stage, the changes in the expression profile of the bFGF, TGF-β, growth differentiation factor (GDF)-5, GDF-6, GDF-7, PDGF, IGF1, and VEGF were reported.[37] It has been reported that miRNA-mediated inhibition of the expression of TGF-β1 and COL1 results in inhibiting tendon differentiation.[36] It is also reported that the miRNA-mediated inhibition of TGF-β2 causes a reduced tenogenic differentiation and tendon injury healing, as it fails to achieve an appropriate amount of collagen and ECM production.[37,55] The relation between early growth response-1 (EGR-1) regulated tenocytes and induced expression of tendon-specific marker genes (SCX, COL1, TNMD, and TNC) has been already established by researchers.[37,40,56] The high-mobility AT-hook 2 (HMGA2) is known for its involvement in many cellular processes, resulting in differentiation along with oncogenesis.[57] However, the study done by Sun and coworkers revealed that HMGA2 plays a critical role in regenerating tendons and maintaining homeostasis.[41,58] Several studies claim the significant role of Rho-associated coiled-coil containing protein kinase 1 (ROCK1) in cell morphology, mitosis, motility, and even senescence, and studies revealed that miR-135a specifically binds to the 3'-UTR of ROCK1,[41,59,60] which results in suppressed proliferation, migration, and tenogenic differentiation.[29,41,61,62] Similarly, the miR-140-5p has been also reported responsible for delayed senescence in human tendon stem/progenitor cells (TSPCs) by targeting peptidyl-prolyl cis/trans isomerase NIMA-interacting 1 (PIN1).[28,41] Therefore, overexpression of the PIN1 may involve senescence and increase tendon differentiation. Other proteins such as p16 and transducer of ERBB2, 1 (TOB1) are also involved in tenogenic differentiation.[63-65] It has been observed, that multiple miRNAs can be deployed to regulate the cellular function to regenerate the tendon through the proliferation and differentiation of tendon cells (Fig. 1). Understanding the role of miRNA combination in tendinopathy and tendon healing will be a decisive factor in the development of new therapeutics.

PPI Network Analysis

To obtain insights into the functional interactions of proteins during tendon healing, the PPI network was created

Table 4. Top Regulated Genes in Human Tendinopathy Based on STRING Analysis

No.	Gene name	Gene symbol
1	A disintegrin and metalloproteinase domain 12	ADAM12
2	Adaptor-related protein complex 3, mu 1 subunit	AP3M1
3	ADP-ribosylation factor-like 7	ARL7
4	Actin related protein 2/3 complex, subunit 5	ARPC5
5	Additional sex combs like 1 (Drosophila)	ASXL1
6	ATPase, H+ transporting, lysosomal 70 kDa	ATP6V1A
7	Beta-site APP-cleaving enzyme 2	BACE2
8	Basonuclin 2	BNC2
9	Carbonic anhydrase XII	CA12
10	Collagen triple helix repeat containing 1	CTHRC1
11	Development and differentiation enhancing factor	DDEF1
12	Dedicator of cytokinesis 10	DOCK10
13	Forkhead box P1	FOXP1
14	G protein-coupled receptor 161	GPR161
15	Insulin-like growth factor binding protein 3	IGFBP3
16	Interleukin 13 receptor, alpha 2	IL13RA
17	Integrin-linked kinase	ILK
18	IQ motif containing GTPase activating protein 1	IQGAP1
19	Integrin, beta 1	ITGB1
20	Inositol 1,4,5-trisphosphate 3-kinase B	ITPKB
21	Jagged 1 (Alagille syndrome)	JAG1
22	Potassium voltage-gated channel 4	KCNE4
23	Laminin, alpha 4	LAMA4
24	Leucine rich repeat containing 15	LRRC15
25	Leucine rich repeat containing 17	LRRC17
26	Myristoylated alanine-rich kinase C substrate	MARCKS
27	Notch homolog 3 (Drosophila)	NOTCH3
28	Protein disulfide isomerase-related	PDIR
29	Periostin, osteoblast specific factor	POSTN
30	RAP1, GTP-GDP dissociation stimulator 1	RAP1GDS1
31	S100 calcium binding protein A10	S100A10
32	SIN3 homolog A	SIN3A
33	Solute carrier family 2 13	SLC2A13
34	TAO kinase 1	TAOK1
35	Tight junction protein 1	TJP1
36	Tenascin C	TNC
37	Ubiquitin-conjugating enzyme E2E 3	UBE2E3
38	WD repeat domain 1	WDR1
39	WNT1 inducible signaling pathway protein 1	WISP1

Table 5. Prediction of the Pathways Influenced or Regulated by the miR-135 and miR-140, a Target-Based Prediction of Biological Function Regulated by Selected miRNAs (miR-135 and miR-140)

miRNA	Target	Function	Inference
miR-135	HOXA2	Upregulated osteogenesis and negatively regulated the HOXA2.	miR-135 positively regulated osteogenic differentiation. miR-35/HOXA2/Runx2 pathway may aid in this regulation.
	HIP1	Worked as an antiangiogenic miRNA under diabetic conditions. Targeting HIP1 to induce VEGF-mediated activation of P38	Therapeutic neutralization of miR-135 under diabetic conditions helped to overcome angiogenic resistance.
	LATS2	miR-135a aided in wound healing by downregulation of LATS2, thereby increasing cell influx	hAMSC derived-exosomal miR-135a promoted wound healing.
	H1F1AN	By directly targeting H1F1AN, miR-135-5p was shown to promote osteoblast differentiation.	miR-135 plays a role in osteogenic differentiation and holds therapeutic potential.
	Unknown targets	miR-135 was observed to be upregulated during myogenic differentiation.	miR-135 may direct the IRS/AKT/PI3K pathway and promote myogenesis.
	ROCK1	miR-135 may downregulate the expression of ROCK1, thereby significantly reducing senescence.	miR-135 serves as a senescence suppressor partly by its activity towards ROCK1.
	Wnt/β-catenin signalling	miR-135 in part interacts with Wnt/β-catenin signaling to work as a tumor suppressor.	miR-135 holds therapeutic potential for breast cancer owing to its anti-tumor potential.
	WISP1 IGFB5	miR-135 in part regulates the expression of several ECM-associated genes including WISP1 and IGFB5.	miR-135 helps to regulate several genes that are essential for ECM, thereby aiding in iPSC generation.
	PIM2	miR-135 negatively interacted with the cell viability and inhibited apoptosis. Also, it regulated PIM2 in a negative regulatory manner.	miR-135 showed potential by working as a negative regulator of PIM2 both, in turn, affected the viability and apoptosis of cells.
	JAK2/STAT3	Upregulation of miR-135 was found to interact with JAK2/STAT3 pathway, which disrupted the Astrocyte mediated neuro-inflammation in turn inhibiting BCP.	miR-135 should be considered a potential treatment target for BCP.
	IRS2	miR-135 was significantly upregulated in the db/db mice GSM and inhibited glucose uptake by targeting IRS2.	IRS2 is a target site for miR135, through which it interferes with insulin signaling and its upregulation in db/db GSM signifies its therapeutic potential.
	RUNX2	miR-135b-5p reduced cell viability and promoted apoptosis in the cell line. However, this was reversed by RUNX2 over-expression.	Study suggests miR-135 inhibits osteogenic differentiation by targeting RUNX2.
	SP1	TGF-β1 promotes upregulation of MSC exosome-derived miR-135b, which can downregulate SP1.	Cartilage repair can be promoted by TGF-β1 regulation of MSC exosome derived 135b, which in turn downregulates SP1.
	SMAD5	miR-135b was significantly upregulated in MM BM hMSC samples, thereby disrupting the osteogenic potential.	miR135 inhibitors in MM-hMSC hold therapeutic potential for resolving the impaired osteogenic capacity and bone formation.
	RUNX2	miR-135a-5p possessed the ability to inhibit osteogenic differentiation via its regulatory action on RUNX2.	Postmenopausal osteoporosis women had high levels of miR-135a-5p and showed to inhibit osteogenic differentiation through its action on RUNX2.
miR-140	PIN1	miR-140-5p regulates PIN1 at a translational level aiding in TPSCS aging.	miR-140-5p and its PIN1 regulation is a viable target for the prevention of TPSC aging.
	Regulated by H19	Overexpression of H19 in TDSC promoted RCT recovery via the miR-140-5p/VEGFA axis.	H19 targets VEGF via regulating miR-140-5p expression during tenogenic differentiation in RCT recovery, suggesting this regulatory pathway can be used for finding novel therapeutics.
	TL4	miR-140-5p was shown to induce target regulation of TL4 and knockdown of TL4 improved tendinopathy.	Overexpression of miR-140-5p lead to inhibition of MyD88 and NF-κB through its regulatory action on TL4, making it a potent target for tendinopathy treatment.
	miR-140 dysregulation	miR-140-3p significantly declined in RCT compared to controls.	miR-140-3p can serve as a potent biomarker for RCT tendinopathy.
	ADAMTS-5	miR-140 directly suppresses ADAMTS-5 levels and negatively regulates it.	miR-140 is a chondrocyte differentiated miRNA and shows reduced expression in osteoarthritis cartilage and ADAMTS-5 was increased, indicating abnormal catabolic/anabolic response in osteoarthritis.
	STAT3	miR-140-5p interacts with STAT3 as shown. The aberrant expression of the former reduced the levels of the latter.	miR-140-5p and STAT3 play an important role in RA FLAS, as miR-140 was shown to suppress proliferation and promote apoptosis in RA FLAS through its activity on STAT3.

Table 5. Continued

miRNA	Target	Function	Inference
	Treatment using TGF-β1	Mechanical loading lowered the levels of miR-140. In converse, the treatment by TGF-β1 resulted in a significant increase in levels of miR-140.	Successful overviewed expression levels of various miRNA under treatment/loading conditions.
	SIRT3	miR-140-3p holds a negative regulatory relation with SIRT3 shown by increased apoptosis of SF in RA.	miR-140-3p promoted the apoptotic activity of SF and reduced cell viability of SF samples via its activity through SIRT. The reverse was observed by its inhibition.

miRNA: microRNA, HOXA2: homeobox a2, HIP1: huntingtin interacting protein 1, VEGF: vascular endothelial growth factor, LATS2: large tumor suppressor kinase 2, hAMSC: human amniotic mesenchymal stromal cells, HIF1AN: hypoxia-inducible factor 1-alpha inhibitor, ROCK1: Rho-associated coiled-coil containing protein kinase 1, Wnt/β-catenin: wingless/integrated/β-catenin, WISP1: WNT1 inducible signaling pathway protein 1, IGFB5: insulin-like growth factor-binding protein-5, ECM: extracellular matrix, PIM2: serine/threonine-protein kinase pim-2, JAK2: Janus kinase 2, STAT3: signal transducer and activator of transcription 3, BCP: bone cancer pain, IRS2: insulin receptor substrate 2, GSM: glucose-sensing module, RUNX2: runt-related transcription factor 2, SP1: specificity protein 1 transcription factor, TGF-β1: transforming growth factor beta 1, SMAD5: mothers against decapentaplegic homolog 5, MM: multiple myeloma, BM: bone marrow, PIN1: peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, TPSC: tendon progenitor stem cell, H19: H19 imprinted maternally expressed transcript, TDSC: tendon derived stem cells, RCT: rotator cuff tear, VEGFA: vascular endothelial growth factor A, TL4: toll-like protein 4, NF-κB: nuclear factor kappa B, ADAMTS-5: A disintegrin and metalloproteinase with thrombospondin motifs 5, RA-FLAS: rheumatoid arthritis-fibroblast-like synoviocytes, SIRT3: Sirtuin 3, SF: synovial fluid, RA: rheumatoid arthritis.

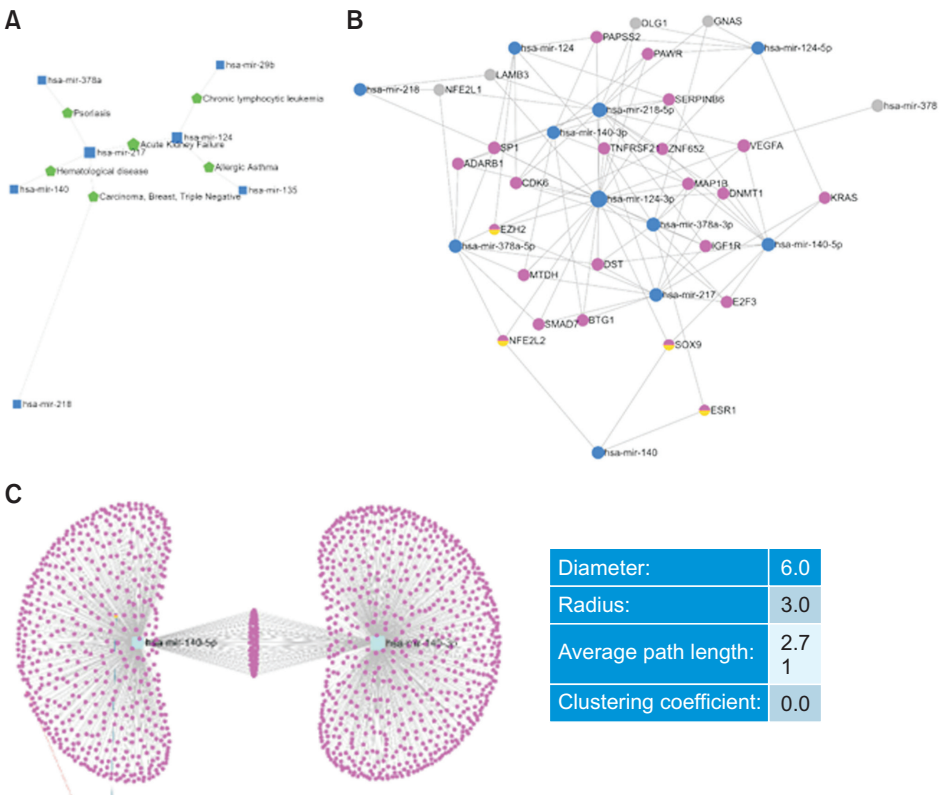

Fig. 4. Network analysis of microRNA (miRNA) interaction using Steiner forest network (A), minimum network algorithm (B), and network analysis on miR-135 and mir-140 (C). (A) Involvement of miRNAs used in this study and their relation in specific disease. (B) Analysis of the miRNAs and related protein interactions and their interconnections that have an impact on epigenetic regulation. (C) Overall impact on different proteins while regulating miR-140 and miR-135 on cellulation function.

from the STRING database (https://string-db.org).[23] To seek potential interactions between responsible proteins regulated by the candidate miRNA, such as ROCK1, HMGA2, p16, TOB1, PIN, EGR1, BIM, Scx, and TNMD, this representative pathway protein was found to be an interactive relation among these (Fig. 3).

Based on the involvement of the miRNA in tendinopathy, the miR-135, miR-124, miR-217, miR-1792, miR-140, miR-218, miR-217, miR-29b-3p, miR-29b, miR17-92, miR-378a, let-7, miR-217, miR-218, and let-7 were further evaluated for their biological role in tendon biology (Table 3). It was noted that the contribution of the different signaling pathways may also contribute significantly during the healing processes. These pathways may influence the tendon healing process by affecting the metabolic process and cell cycle via cytokine–cytokine receptor interaction. The interaction between two or more proteins through non-covalent bonding (PPI interaction) plays a significant

role in the biochemical process. This PPI study allows for the identification of the key protein in response to the selected miRNA. The relationship between various key proteins is shown in Fig. 3. Cellular factors of individual patients play an important role in the healing process. The protein expression changes and their interaction with other proteins provide an insight into the molecular mechanism of tendinopathy. In this study, 40 proteins were identified for their key role in tendinopathy (Table 4). Among those nine important proteins selected for the PPI network, these were Scx, TNMD, ROCK1, PIN1, TOB1, P16, HMGA2, ERG1, and BIM (Fig. 3). The study revealed that out of all selected miRNAs (miR-29b-3p, miR-29b, miR17-92, miR-135a, miR-124, miR-217, miR-140-5p, miR-378a, let-7, miR-218), the miR-135 and miR-140 were found to be potential candidates for therapeutics development against degenerative tendinopathy. The roles of miR-135 and miR-140 are summarized in Table 5. It was found that miR-135 was mainly involved in the negative regulation of IL-17 (cytokine) expression, an important cytokine for inflammatory responses. However, all predicted miRNAs have a certain potential to be considered for therapeutic development in combination (Fig. 4). This study mainly focused on cell proliferation and suppression of senescence. Therefore, miR-135 and miR-140 were selected to validate the effect on degenerative tendinopathy (Fig. 4).

miRNA-Gene Correlation

After analysis of the network, top genes were shortlisted (based on greater correlation with the miRNAs). The poorly correlated genes were omitted from this study to get a precise target. The selected namely TGF-β1 and COL1A1, BIM, Scx and TNMD, ROCK1, EGR1, PIN1, HMGA2, p16, and TOB1 were found to be prevalent in tendinopathy. Further, it was also reported to be involved in tendon healing.[66] The tenogenic key proteins, especially growth factors, namely IGF-1, TGF-β, PDGF, VEGF, bFGF, and GDF along with matrix proteins involved in matrix turnover, were analyzed by KEGG tool. Fig. 5 shows the relation of the used miRNAs with the pathway and their effect on laboring cell/conditions. These miRNAs establish a correlation with osteoarthritis while other conditions are related to colorectal cancer. Therefore, targeting these miRNAs will be safer. It is also anticipated

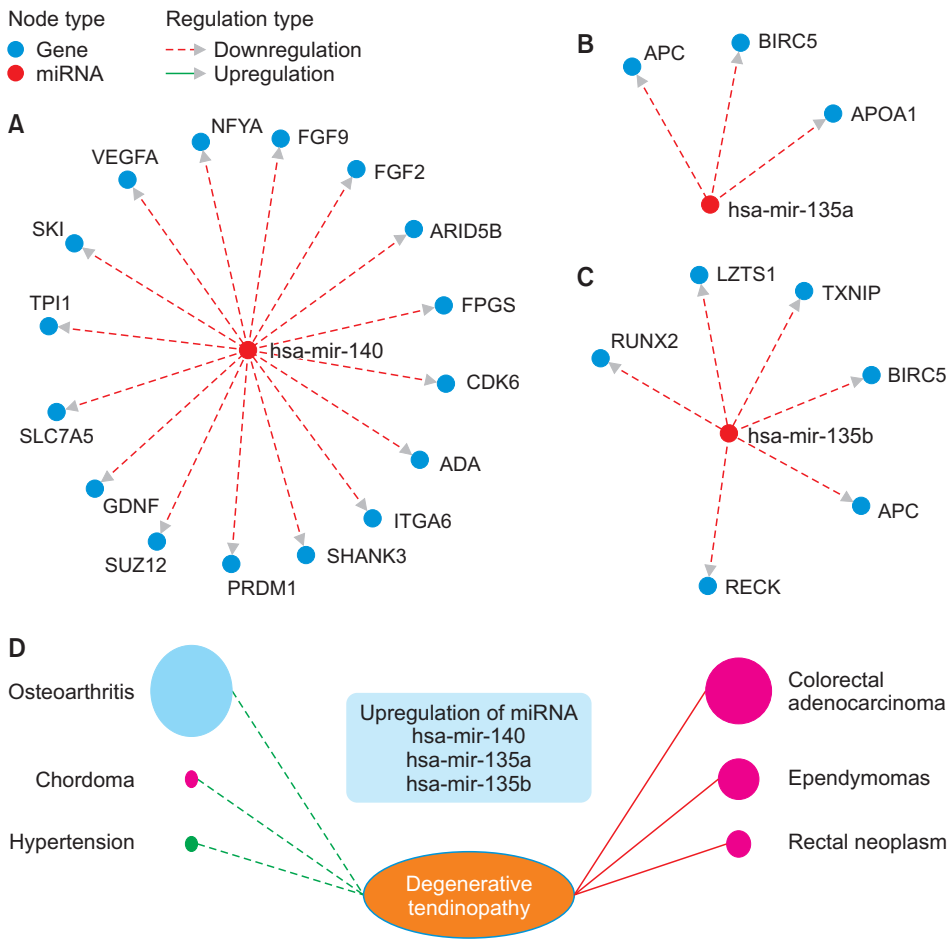

Fig. 5. Predicted relationship between the role of selected microRNAs (miRNAs) in tendinopathy and significant genes to be downregulated in response to overexpression of miR-140 and miR-135. These miRNAs were also reported in other diseases than predicted tendinopathy. Network analysis of miRNA interaction in disease development. (A) has-mir-140. (B) has-mir-135a, (C) miR-135b. (D) Involvement of predicted miRNA in other diseases.

that a combination of these miRNAs with other tissue-specific miRNAs will help reroute the cellular phenomena to combat the degenerating disease.

DISCUSSION

miRNAs are key epigenetic regulators. These endogenous non-coding RNAs regulate the gene expression post-transcriptionally. To date, a huge number of miRNAs have been reported with their role in various cellular phenomena. Prediction and identification of miRNAs and their respective target genes in this study revealed an insight into epigenetic regulation of cellular reprogramming and senescence. This information could potentially be used for the development of a new class of therapeutics for degenerative diseases such as tendinopathy and osteoarthritis.

Based on seed matches, a set of miRNAs were derived from Targetscan. However, most of the predicted miRNAs were found to be involved in various pathways, i.e., one miRNA can regulate the expression of many genes while many miRNAs can regulate the expression of one gene. Therefore, the selection of more precise miRNAs was the main aim of the study. Our focus was to predict a single miRNA or combination of miRNAs, which could reprogram the resident cells in a proliferative state and subsequently differentiate in tenocytes. The healing process of the tendon has various stages, including inflammation, proliferation, differentiation, and matrix remodeling. However, all the cellular reprogramming in degenerative tissue is crucial; therefore, the prediction was mainly focused on miRNAs that could induce sustainable proliferation of resident cells. The selected miRNAs against targeting key regulatory genes involved in tendinopathy are provided in Table 3. These miRNAs were subject to further screening to find the most suitable candidates. Strong pairing is one of the selection criteria to ensure the perfect Watson-Crick pairing between miRNAs, and mRNA only 7mer-m8 seed match was considered. Another exclusion screening criterion was involvement in non-desirable pathways. Among all studied pathways and key genes involved in the biology of tendons, we have target ROCK-1 and PIN-1 pathways governing mitosis (cell division) and senescence, respectively.[28,41,59,60] It was revealed by GO analyses (Table 3) that miR-135 and miR-140 were found to be involved in the regulation of ROCK1, VEGF, and TGF-β. The study also pointed out the involvement of miRNA-135 in the regulation of PDGF, IGF, and bFGF. However, GDF was found to be regulated by miR-140. The mentioned proteins were reported as key regulators for tendon healing (Fig. 1). The role of miRNA-135 and miRNA-140 in cell proliferation and differentiation, as well as in remodeling of the ECM, was also established by previous researchers. The properties that make miRNA-135 and miRNA-140 as primary choices for this study are also described in Table 5. All mentioned pathways were reported to be involved in tendinopathy. Regulating the mentioned key pathways that lead to cellular proliferation and tenogenic differentiation could be an approach to treating degenerative tendinopathy. Therefore, the synergic effect of two miRNAs was evaluated in this study as a model to predict the role of various combinations of miRNAs to achieve an inclusive approach for the treatment of degenerative tendinopathy. Furthermore, the miRNA–gene regulatory network allows us to understand the molecular modulation of tendon healing for the development of new combinatorial therapy using miR-140 and miR-135 information. The target gene is primarily responsible for cell proliferation and differentiation, thus finally an adequate number of tenocytes will remodel the ECM. KEGG analysis also showed that the targeted protein is also involved in cell proliferation and differentiation. Thus, dually targeting miRNA will have better potential to regenerate the tendon tissue.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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SUPPLEMENTARY MATERIAL

Supplementary material is available in the electronic version of this paper at the CiOS website, www.ecios.org

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