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Research article

FUNCTIONAL CHARACTERIZATION OF HUMAN KINDLIN-2 CORE PROMOTER IDENTIFIES A KEY ROLE OF SP1 IN KINDLIN-2 TRANSCRIPTIONAL REGULATION

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Abstract: Kindlin-2 is a recently identified FERM and PH domain containing integrin interacting protein. Kindlin-2 is ubiquitously expressed in normal tissues. So far, much effort has been spent exploring the functional aspects of Kindlin-2. However, the transcriptional regulation of Kindlin-2 has not yet been investigated. In this study we identified and functionally characterized the promoter of the human Kindlin-2 gene. We show that the core promoter of Kindlin-2 is a 39 base pair long GC rich fragment located -122/-83 upstream of the Kindlin-2 transcription start site. Functional characterization of this core promoter region by both *in silico* as well as *in vitro/in vivo* analysis shows that the transcription factor SP1 plays an important role in regulation of Kindlin-2 expression.

Key words: Kindlin-2, FERMT2, PLEKHC1, Mig-2, Transcription factor SP1, Promoter, Transcription start site, Gene regulation, *Cis*-acting elements, CpG island, Cell migration, Integrin, Gene expression

INTRODUCTION

Kindlin-2, FERMT2, PLEKHC1, Mig-2 belongs to the Kindlin family of proteins. Evolutionarily, Kindlin-2 is the most conserved protein within the Kindlin

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Abbreviations used: DBTSS – database for transcription start site; FERM – 4.1 protein, ezrin, radixin and moesin; Mig-2 – mitogen-induced gene-2; PH –pleckstrin homology

family [1]. This family consists of three members in human and mice: Kindlin-1, Kindlin-2 and Kindlin-3. All three Kindlin proteins are composed of a centrally located FERM (Band 4.1/Ezrin/Radixin/Moesin) domain interrupted by a pleckstrin homology domain [2]. Kindlin-2 localizes to focal adhesions and directly binds to the tail domain of β1 and β3 integrins [3]. Kindlin-2 also interacts with migfilin, a filamin and vasodilator stimulated protein (VASP)-binding protein. Kindlin-2 requires integrin-linked kinase [4] for its localization at focal adhesions [5] and in turn recruits migfilin to focal adhesion sites [6]. Due to its essential role in integrin activation, Kindlin-2 is involved in many important physiological processes, including heart development [7], cell migration [6] and cancer progression [8].

The expression patterns differ between the three Kindlins. For example, Kindlin-1 is predominantly expressed in the epidermis and only weakly expressed in the dermis. Similarly, Kindlin-3 expression is restricted to hematopoietic cells, where it is the dominant form of Kindlin. However, Kindlin-2 is ubiquitously expressed in most tissues [9]. The differential expression pattern of the Kindlins may be one of the reasons for the distinctive phenotypes resulting from the loss of different Kindlins. For example, the Kindlin-2 homolog in C. *elegans*, UNC-112, is essential for embryonic development [7]. Also, loss of Kindlin-2 in mice results in pre-implantation embryonic lethality and knock-down of Kindlin-2 in zebrafish reveals a strong relationship between cardiac development and the function of Kindlin-2 [10]. Consistent with the restricted expression of Kindlin-3 in hematopoietic tissues, mice lacking Kindlin-3 show severe osteoporosis, hemorrhage and defects in the erythrocyte membrane skeleton, and die within one week after birth [11, 12]. In addition, mutation in Kindlin-3 in human leads to immune and bleeding disorders [13].

However, the transcriptional regulation of the Kindlin family has remained unknown. In the present report, we have identified and characterized both *Cis* and *Trans* elements involved in the regulation of Kindlin-2 expression, which may contribute to the expression profile of Kindlin-2 in different tissues.

MATERIALS AND METHODS

In silico analysis

The genomic sequence of the upstream flanking region of the Kindlin-2 gene (Entrenz gene ID 10979) was retrieved from the NCBI human genome database. To search for the experimentally verified transcription start sites, the online database *Database of transcription start sites* (DBTSS) was employed by using the Refseq sequence NM_ 006832 (http://dbtss.hgc.jp) [14]. In order to determine the putative binding sites for transcription factors, online tools *Consite* (http://asp.ii.uib.no:8090/cgi-bin/ CONSITE/ consite) and *TFSEARCH* (www.cbrc.jp/research/db/TFSEARCH.html) were used [15]. Comparative sequence analysis was done by using the *GenomeVISTA* tool (http://genome.lbl.gov/cgi-bin/GenomeVista) [16].

Cell culture

African green monkey kidney cell line COS7, Mouse fibroblast NIH 3T3 and C3H10T(1/2) and the human epithelial cervical cell line HeLa cells were cultured in DMEM while human prostate cancer cells PC3 were cultured in RPMI-1640. The medium was supplemented with gentamicin (10 µg/ml), L-glutamine (2 mM) and 10% fetal calf serum (GIBCO) and maintained at 37^{o}C and 5% CO_2 in a humidified incubator.

Cloning of Kindlin-2 promoter and construction of deletion mutants

The putative promoter for Kindlin-2, a 1615 bp long genomic fragment, was amplified by PCR using human genomic DNA (kindly provided by Alamdar Baloch, Dept of Laboratory Medicine, Karolinska Institutet) and a primer set as shown in Tab. 1 (with the transcription start site identified by using DBTSS designated as +1). The product was cloned upstream of the *firefly* luciferase gene in pGL-4.21 (Promega, USA). Progressive 5′-end deletions of Kindlin-2 promoter were performed by PCR amplification by using primer sets as shown in Tab. 1. All constructs were isolated with Jetquick maxi prep plasmid extraction kit (Genomed, USA) and confirmed by DNA sequencing (Macrogen, South Korea).

Tab. 1. Nucleotide sequences of the primers used for making the deletion constructs of Kindlin-2 promoter.

Symbol	Sequence
P (-1615/+15)-F	GCctcgagCCTCATCTCCGGACATTCAT
P (-1615/+15)-R	CATaagcttTGGCCGGAGCGGCTAATGGAGT
P (-1000/+15)-F	GCctcgagCACCTCTCACCTCCCTGAGGCTT
P (-1000/+15)-R	CATaagcttTGGCCGGAGCGGCTAATGGAGT
P (-600/+15)-F	GCctcgagCGGGTCCTTCATTTCCATAA
P (-600/+15)-R	CATaagcttTGGCCGGAGCGGCTAATGGAGT
P (-300/+15)-F	GCctcgagCGGGTCCTTCATTTCCATAA
P (-300/+15)-R	CATaagcttTGGCCGGAGCGGCTAATGGAGT
P (-145/+15)-F	GCctcgagAGGGCAGCTCTGCGGGCGGCGAA
P (-145/+15)-R	CATaagcttTGGCCGGAGCGGCTAATGGAGT
P (+15/+90)-F	GCctcgagACTCCATTAGCCGCTCCGGCCA
P (+15/+90)-R	CATaagcttTGGCCGGAGCGGCTAATGGAGT

Small letters represent sites for restriction enzymes Xho1 and Hind111. F: Forward, R: Reverse

Transfection and luciferase assays

Cells were plated in 24-well plates and incubated overnight. All the constructs for Kindlin-2 promoter luciferase vectors were transfected into cells with 0.6 µl Fugene-6 and 200 ng plasmid DNA per well, following the manufacturer's instructions. In all transfections, *Renilla* luciferase expression plasmid, pRL-SV40 (Promega, USA), was co-transfected as a transfection control (20 ng

per well). The cells were cultured at 37°C for 48 h. Luciferase activities were measured by the Dual Luciferase Reporter Assay system (Promega, USA). For serum treatment, 4 h before the transfection, cells were starved with 0% serum-free medium and incubated for 72 h and then treated with 10% serum. Luciferase assay was done at different time intervals after the serum treatment.

SP1 inhibitor treatment

SP1 inhibitors mitoxantrone and daunorubicin (Sigma-Aldrich, USA) were reconstituted in PBS buffer and 50 or 100 nM mitoxantrone or 200 nM daunorubicin was added immediately after cell transfection with the Kindlin-2 promoter constructs and incubated for 72 h.

Knockdown of SP1 by siRNA and wound healing assay

Pre-validated SP1 siRNA and control siRNA were purchased from Qiagen (Qiagen, USA). The siRNA transfection was done with Lipofectamine-2000 (Invitrogen). Briefly, cells were plated in 6-well plates and were transfected with SP1 siRNA (25 nM and 50 nM) or control siRNA (50 nM) on the following day. The medium was changed six hours after the transfection. Cells were harvested after 72 hours. For wound healing assay, PC3 cells were cultured in 6-well plates. 72 h after transfection with SP1 siRNA, a single wound was created in the center of the cell monolayer by scratching with a sterile plastic pipette tip. The debris was removed by washing with serum-free medium. Photographs of cells were taken after given time intervals.

Western blot analysis

Total protein was extracted from mitoxantrone or SP1 siRNA treated and non-treated COS7 and PC3 cells by cell lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 100 mM NaCl, 0.5% Nonidet P-40 and protease inhibitor cocktail (Roche, Germany). 20 μg of protein per lane was resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electro-blotted onto a PVDF membrane (Millipore). Blots were incubated with an affinity purified anti-Kindlin-2 polyclonal antibody (1:1000 dilution) [8] or an anti-tubulin antibody (Santa Cruz). Following incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody at 1:3000 (Jackson Immuno Research, USA), detection was performed with a chemiluminescent substrate kit (PerkElman, USA).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed for the transcription factor SP1. PC3 cells were fixed with 1% formaldehyde, quenched using 1.25 M glycine, resuspended in SDS lysis buffer and sonicated (10 s pulses×5 at 50% duty cycle, output 5, Diagenode sonicator) to generate chromatin sizes between 200 and 1000 bp. 20 μl of soluble DNA fraction was saved as input (10%). 5 μg of anti-SP1 (Santa Cruz) and mouse IgG (Millipore) antibodies were used for the ChIP assay. 2 μl of eluted DNA from ChIP and input reactions was used for PCR with primers -

150F 5′ AGGGCAGCTCTGCGGGCGGCGAA -3′ and +450 ′ AGAGCCATGG CTCCTTCCTGCG -3′ using the program 95°C for 2 min (95°C for 30s, 60°C for 60s and 68°C for 1 min) 40 cycles and 68°C for 7 min. The products were resolved on 1% agarose and were confirmed for specificity by sequencing. Each ChIP assay was repeated twice.

RESULTS

Identification of Kindlin-2 transcription start site (TSS)

We aimed to identify regulatory mechanisms that control Kindlin-2 transcription. To verify the transcription start site (TSS) of Kindlin-2 (Refseq sequence NM_006832), we employed the Database of Transcription Start Site (DBTSS) containing 5 - end cDNAs obtained by the oligo-capping method. The database showed that 14 out of 17 clones of the human Kindlin-2 upstream region shared the same TSS (Suppl. Tab. 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-011-0028-6) starting from 57 bp upstream of the aforementioned Refseq sequence (Fig. 1A). This means that the majority of Kindlin-2 mRNA might be transcribing from this TSS; therefore we designated this site as +1.

5′-flanking fragment of Kindlin-2 gene shows significant promoter activity

To find the potential promoter region of the Kindlin-2 gene, we used UCSC genome browser to explore the upstream region of Kindlin-2. Based upon this *in silico* analysis, we selected a 1615 bp long 5′-flanking fragment of the Kindlin-2 gene which includes a conserved region among different species and the Kindlin-2 TSS. For simplicity, we term this fragment the full-length promoter. Luciferase assay for the promoter showed a 40-fold higher transcriptional activity as compared to the mock vector in COS7 cells, which expressed a high level of endogenous Kindlin-2 (Fig. 1B and 1C).

The major transcriptional regulatory region for Kindlin-2 resides at -122 to -83 upstream of Kindlin-2 TSS

To identify the region necessary for basal transcription of Kindlin-2, a series of deletion mutants was established (Fig. 1B). Luciferase reporter assay for these deletion mutants in COS7 cells revealed that the region encompassing -122 to -83 shows almost the same activity as does the full length promoter. Therefore we term this region the core promoter. The downstream of this region, i.e. -83/+90, did not show any significant activity. We further tested the activity of the core promoter in four other cell lines in which the endogenous Kindlin-2 level was high, i.e. in 3T3, C3H10T(1/2), HeLa and PC3 cells. In all these cell lines, the core promoter showed significant activity (Fig. 1C and 1D). These data demonstrated that the established Kindlin-2 reporter construct might include the major transcriptional regulatory region of Kindlin-2.

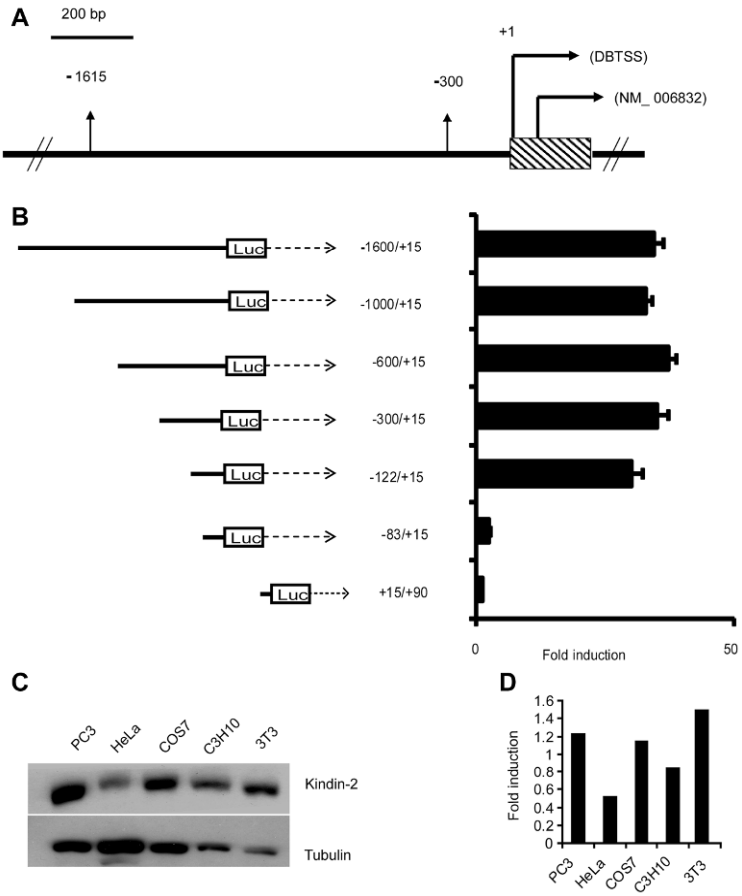

Fig. 1. Promoter activity of the upstream regulatory region of Kindlin-2. A – A schematic diagram of Kindlin-2 promoter. Dashed box represents the first exon. B – Narrowing down of Kindlin-2 full length promoter by making the deletion mutants. Left panel: A schematic representation of the Kindlin-2 promoter deletion constructs. The solid bars represent the deletion constructs while the open boxes represent the luciferase gene. Right panel: Promoter activity of different deletion constructs. The plasmids containing sequentially deleted fragments of human Kindlin-2 promoter (-1615/+15) were transfected into COS7 cells. The values represent fold changes in promoter activity as compared to the empty mock vector pGL-4.21, normalized to *Renilla* luciferase (pRL-SV), which acts as an internal control. The values are $\pm$ SEM of at least three independent experiments, done in triplicate. C – Western blot analysis of expression of endogenous Kindlin-2 protein in all the cell lines shows high endogenous expression of Kindlin-2 protein. Cells were harvested and lysed 72 h after cell culturing. Western blot analysis was done by using anti-Kindlin-2 and anti-tubulin antibody. D – The Kindlin-2 core promoter region shows high activity in the cells in which endogenous Kindlin-2 expression is high. The values represent the fold change in promoter activity as compared to the empty mock vector pGL-4.21, normalized by *Renilla* luciferase (pRL-SV), which acts as a transfection control. The values are $\pm$ SEM of at least three independent experiments, done in triplicate.

***In silico* analysis of core promoter shows numerous SP1 binding sites with interesting conservation pattern**

To fully characterize the core promoter region, we scanned this region by using the online tool *CONSITE* to find the putative transcription factor binding sites. The *in silico* analysis revealed that the region -122/-83 is highly GC rich and does not contain any TATA box. Interestingly, numerous SP1 transcription factor binding sites were predicted in this region (Fig. 2A). Although this region did not display significant conservation between different species, the presence of SP1 binding sites was highly conserved in most of the species (Suppl. Fig. 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-011-0028-6). In order to elucidate whether the promoters of the other Kindlins, Kindlin-1 and Kindlin-3, may contain SP1 binding sites, we analyzed the potential presence of putative SP1 binding sites in a 1 kb region upstream of both of these paralogs. Interestingly, no SP1 binding site was found in either of the two paralogs, showing that SP1-dependent promoter regulation might be Kindlin-2 selective (data not shown).

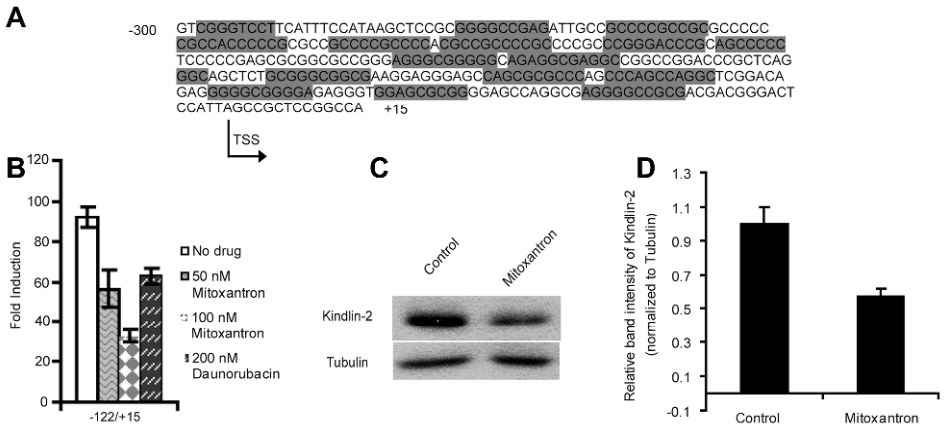

Fig. 2. Characterization of core promoter. A – Nucleotide sequence of the Kindlin-2 core promoter. The shaded areas represent putative SP1 binding sites. B – Effect of SP1 inhibitors on Kindlin-2 promoter activity. COS7 cells were transfected with pGL-4.21 vector containing the 39 bp promoter fragment (-122/+15) which contains numerous SP1 binding sites. The SP1 inhibitors mitoxantrone and daunorubicin were added in given concentrations at the same time as transfection. Cells were harvested 48 h after transfection and analyzed for luciferase activity. The *firefly* luciferase values are normalized by *Renilla* luciferase that acts as transfection control. The values are $\pm$ SEM of at least three independent experiments, done in triplicate. C – Effect of mitoxantrone on endogenous Kindlin-2 expression. COS7 cells were treated with 200 nM mitoxantrone and incubated for 72 h at 37^{o}C. Western blot analysis was done by using anti-Kindlin-2 and anti-tubulin antibodies. (D) The relative band densities of Kindlin-2 normalized to alpha-tubulin. The control values were brought to 1.

SP1 is involved in the regulation of Kindlin-2 transcriptional regulation

To experimentally examine the role of SP1 on the regulation of Kindlin-2 promoter activity, we used the SP1 inhibitors mitoxantrone and daunorubicin. Interestingly, treatment of COS7 cells with these two inhibitors drastically reduced both the activity of the Kindlin-2 promoter and the endogenous Kindlin-2 expression. Similarly, SP1 knockdown (Fig. 2B-2D) by siRNA showed significant downregulation of endogenous Kindlin-2 protein expression both in COS7 and PC3 cells (Fig. 3A-3F). Together, these results show that SP1 plays a key role in controlling Kindlin-2 expression. In order to verify whether the effect of SP1 on endogenous Kindlin-2 expression may be due to direct binding of SP1 on the Kindlin-2 promoter, we performed a ChIP experiment (Fig. 3G).

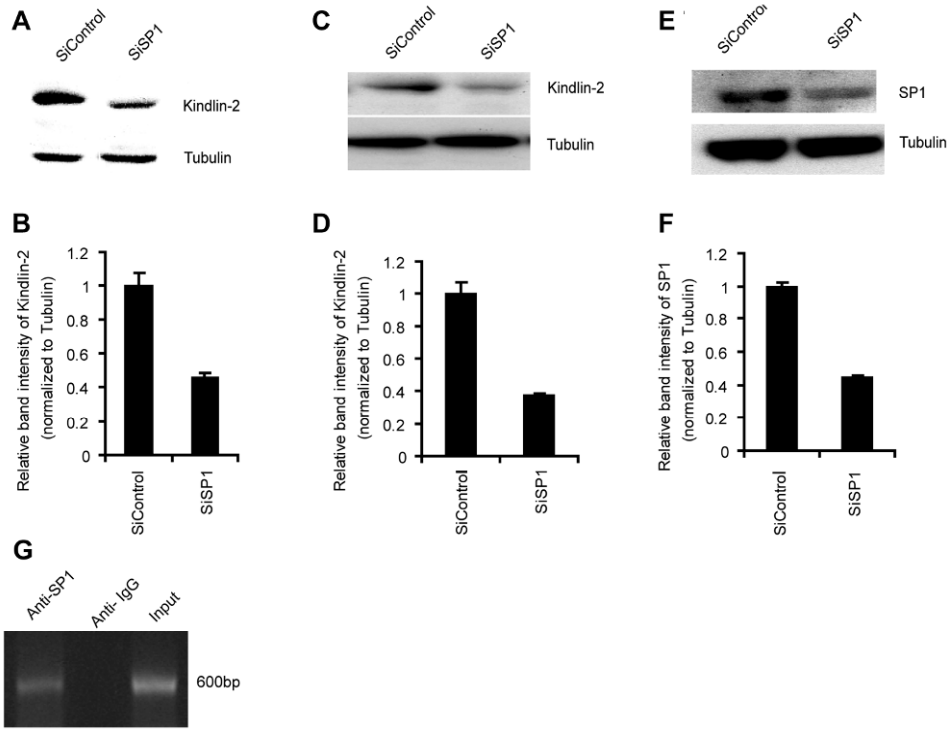

Fig. 3. Regulation of Kindlin-2 expression through SP1. A, C – Effect of SP1 knockdown by SP1-siRNA in COS7 and PC3 cells respectively. COS7 and PC3 cells were treated with SP1 siRNA and control siRNA and incubated for 72 hours. Western blot analyses were done by using anti-Kindlin-2 and anti-tubulin antibodies. E – Significant knockdown of SP1 by SP1 siRNA. B, D and F – The relative band densities of Kindlin-2 (B, D) and SP1 (F) normalized to alpha-tubulin. The control values were brought to 1. G – ChIP analysis. PC3 cells were used for ChIP assay. The oligos used for ChIP assay are described in Materials and Methods. Lane 1, PCR product from DNA template of Kindlin-2 promoter immunoprecipitated by anti-SP1 antibody; lane 2, PCR product from DNA template of Kindlin-2 promoter immunoprecipitated by anti-IgG antibody; lane 3, PCR product derived from direct input DNA template without immunoprecipitation.

ChIP results showed that SP1 does bind with the Kindlin-2 core promoter region. Our results thus suggest that SP1-dependent regulation of Kindlin-2 expression is caused by direct binding of SP1 to the Kindlin-2 promoter.

Kindlin-2 core promoter is not responsive to serum starvation

Kindlin-2 was initially named Mig-2 (Mitogen-induced gene-2) because it displays very early induction after serum treatment of cells [17]. Therefore, we examined whether the Kindlin-2 core promoter is induced by serum stimulation. However, we did not find any change in activity in the Kindlin-2 core promoter when starved cells were treated with serum, showing that the Kindlin-2 core promoter, which is regulated by SP1, is not responsive to serum induction (Fig. 4A-B).

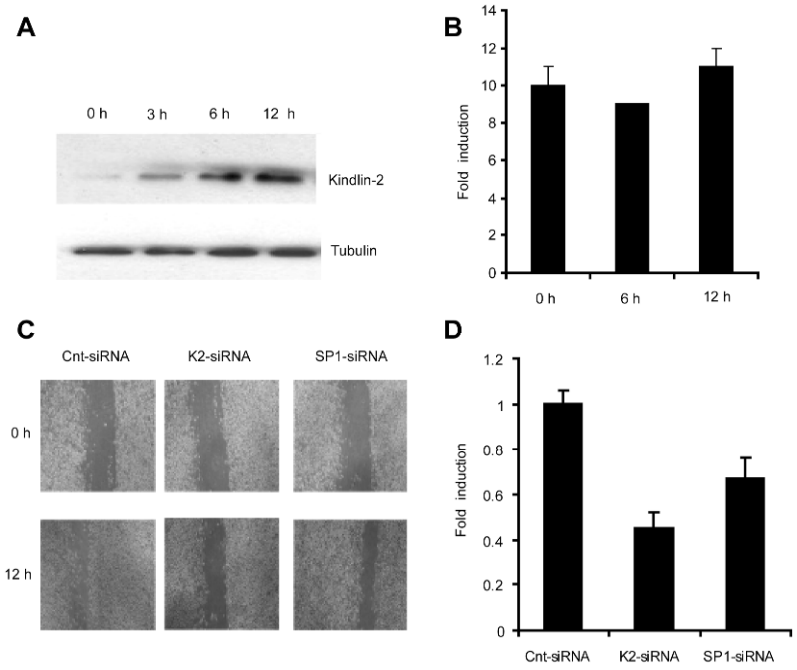

Fig. 4. Effect of serum induction on Kindlin-2 expression and cell migration assay. A – Cells were serum starved for 72 hours and then treated with 10% FBS. Western blot analysis was done by using anti- Kindlin-2 and anti-tubulin antibodies. B – The plasmids containing the Kindlin-2 core promoter fragment (-122/+15) were transfected into COS7 cells. The values represent the fold change in promoter activity as compared to the empty mock vector pGL-4.21, normalized by *Renilla* luciferase (pRL-SV), which acts as an internal control. The values are $\pm$ SEM of at least three independent experiments, done in triplicate. C – Wound healing assay. Wounds were made by a pipette tip on confluent PC3 cells treated with control and SP1 siRNA and allowed to heal for 12 h. The microscopic photos were taken under ×10 magnification. D – For quantifications, the area covered by the cells after incubation for 12 h was measured using Image J. The control values were brought to 1.

SP1 may play a part in regulating Kindlin-2 function

Kindlin-2, being an important integrin interacting protein, has been implicated in cell migration [8]. In order to test whether the transcriptional regulation of Kindlin-2 through SP1 may have physiological importance, we explored the effect of SP1 regulated Kindlin-2 expression in cell migration. The wound healing assay showed that knocking down of SP1 leading to reduction in Kindlin-2 expression does affect cell migration although the effect was not as significant as with direct knocking down of Kindlin-2 (Fig. 4C-D).

DISCUSSION

Kindlin-2 is expressed ubiquitously in most tissues, while its two other paralogs, Kindlin-1 and Kindlin-3, are predominantly expressed in epithelial tissues and the hematopoietic system respectively [9]. However, no study has so far been performed on the transcriptional regulation of Kindlin-2. In this study, our main aim was to characterize both *Cis* and *Trans* elements controlling the regulation of Kindlin-2 expression.

We identified and characterized the Kindlin-2 promoter and showed a role for the transcription factor SP1 in Kindlin-2 transcriptional regulation. The functional analysis of the Kindlin-2 promoter region revealed that most of the promoter activity is exhibited by a 39 base pair fragment (-122/-83) in all the cell lines we tested. This is exactly in accordance with what is known for ubiquitously expressed genes: that they tend to have GC rich TATA-less promoters [18]. Comparative sequence analysis for the core promoter region showed that this region is partially conserved in different species, i.e. in monkey, mouse, chicken and zebrafish. However, the presence of SP1 binding sites is highly conserved in these species, with the only exception of zebrafish, which is quite understandable as it is placed at the base of the vertebrate phylum. This is in accordance with the well-established fact that *cis* regulatory sequences diverge more rapidly and extensively as compared to protein-coding portions of the genome. This divergence of regulatory sequences in turn gives rise to large variety of the expression pattern of a given gene [18-21].

In our study, we showed that SP1 plays a pivotal role in the regulation of Kindlin-2 expression. This finding might explain why Kindlin-2 is expressed ubiquitously in different tissues. SP1 itself is a ubiquitously expressed transcription factor and hence likely contributes towards the ubiquitous expression of Kindlin-2 in different tissues [22]. Similarly, absence of SP1 binding sites in both the Kindlin-1 and Kindlin-3 promoters may also explain why these genes are not ubiquitously expressed. The ubiquitous expression of Kindlin-2 can also be explained on the basis of the simple functional structure of the Kindlin-2 promoter. For example, we clearly showed here that almost all the activity of the Kindlin-2 promoter is controlled by a 39 base pair fragment (-122/-83). Besides this region, there seems to be no other region within this 1615 bp fragment controlling Kindlin-2 promoter activity as efficiently as this

core promoter. This means that Kindlin-2 expression might not be controlled by a complex interaction of factors as mainly the interaction between one type of *Cis* and *Trans* elements is adequate for the regulation of its expression. The cell migration assay revealed that transcriptional regulation of Kindlin-2 through SP1 may affect Kindlin-2 function. It will be interesting to see whether SP1 can affect Kindlin-2 function in certain diseases, e.g. in cancer metastasis. Our finding that serum starvation does not affect the activity of the core promoter indicates that the mitogenic inducibility of Kindlin-2 may be due to either some serum response element(s) controlled independently of this core promoter or to post-transcriptional modification.

In conclusion, the 39 base pair long Kindlin-2 core promoter belongs to the highly CpG rich class of promoters. SP1 regulates Kindlin-2 protein expression by directly binding to its promoter. It will be interesting to see if in addition to SP1 other factors regulate Kindlin-2 expression, especially in cancer tissues and in different mitogenic conditions.

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