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Lipocalin 24p3 is regulated by the Wnt pathway independent of regulation by iron

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Abstract Lipocalin 24p3 plays a direct role in iron transport and regulates the levels of important proteins of the iron metabolism. Iron-loaded 24p3 binds to its specific receptor (24p3R) on the cell surface. Upon binding to its receptor, 24p3 is internalized into the cell, where it releases its bound iron. Iron-free 24p3 can withdraw iron from inside the cell to the outside by a reverse mechanism. We analyzed the role of the murine 24p3 gene *Lcn2* (alias *24p3*) as a target of the Wnt pathway. In cells with activated Wnt pathway, the levels of 24p3 protein and RNA were decreased. The withdrawal of iron led to 24p3 downregulation, and iron addition to iron-deprived cells induced 24p3 expression. Despite its strong inhibitory effect on 24p3 expression, Wnt pathway activation had no effect on the intracellular iron level. In cells with nonactivated Wnt pathway, we found an as yet unidentified transcript of 24p3R. Our results indicate independent regulation of 24p3 expression by the Wnt pathway and by the intracellular iron level. Differential splicing of the 24p3R transcript, depending on the activation state of the Wnt pathway, may modify the function of 24p3.

1. Introduction

The neutrophil gelatinase-associated lipocalin (NGAL), or 24p3, belongs to the superfamily of structurally related small extracellular lipocalin proteins (see Kjeldsen et al. [1] for a review). The 24p3 cDNA was originally identified in murine kidney cells transfected with SV40 T antigen [2]. Lipocalin 24p3 plays a role in iron metabolism and iron delivery (see Yang et al. [3] for a review). Transferrin is necessary for iron transport in the serum, but 24p3 may play a role in paracrine transport of iron, which is independent of transferrin [4]. Iron-loaded 24p3 upregulates the cytoplasmic iron storage protein ferritin and downregulates the transferrin internalizing protein TfR (transferrin receptor-1) [4].

Recently, the cell-surface receptor 24p3R was identified [5]. Ectopic 24p3R expression confers on cells the ability to undergo either iron uptake or apoptosis, depending on the iron content of its ligand, 24p3. Iron-loaded 24p3 increases intracellular iron levels, and iron-lacking 24p3 leads to a decrease in intracellular iron levels. This decrease induces the expression of the proapoptotic protein Bim, resulting in apoptosis. 24p3 can induce changes in BAD phosphorylation and repress the antiapoptotic transcription factor ATFx [6,7]. Deprivation of the essential growth factor interleukin-3 (IL-3) leads to induction and secretion of 24p3. Repression of 24p3 by IL-3 is considered necessary for the survival of hematopoietic cells [6]. Because neutrophil granulocytes are sensitive to 24p3-induced apoptosis, 24p3 might be important for the regulation of the number of these cells.

In a previous study, we identified the murine 24p3 gene *Lcn2* (alias *24p3*) as a target gene of the Wnt pathway [8]. In a microarray screen, *24p3* showed the strongest difference among 147 differentially expressed target sequences. The Wnt pathway regulates cell proliferation, differentiation, organogenesis, and oncogenesis (see Giles et al. [9] and He [10] for a review). Wnt-activated cells contain high levels of the protooncoprotein β-catenin in the cytosol and

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the nucleus. Nuclear β-catenin functions as an activating cofactor for transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family. The β-catenin/TCF complex activates the transcription of specific target genes, which are responsible for the biological effects of the Wnt pathway. Wnt target genes and differential gene expression patterns of Wnt activated have been described in several studies. (For a list of direct and indirect Wnt target genes in various organisms and systems, see http://www.stanford.edu/~rnusse/pathways/targets.html; note that the genes are identified as in the sources cited, not necessarily in nomenclature suggested by the Gene database [http://www.ncbi.nlm.nih.gov]).

Here, we present findings about the regulation of the 24p3 lipocalin protein by the Wnt pathway and by iron, to clarify this protein's regulatory mechanism and its cellular function.

2. Materials and methods

2.1. Cell culture

The colorectal tumor cell line SW480 and the rabbit kidney cell line RK13 were cultured under standard conditions recommended by the supplier (ATCC, Manassas, VA). In SW480 cells, the Wnt pathway is constitutively activated by the loss of functional APC. The activation of Wnt-1 expression in murine mammary epithelial C57MG cells results in morphological transformation [11–13]. C57MG/Wnt-1 cells, which have been stably transformed by infection with a retroviral vector containing Wnt-1, have been used for the identification of Wnt targets [14].

Aberrant long-term expression of Wnt-1 might lead to secondary effects, such as activation of feedback loops and downregulation of genuine Wnt targets. To rule out unspecific results, we analyzed different cell models after Wnt pathway activation by various reagents. We analyzed Wnt-1 transfected cells, or cells after incubation with the glycogen-synthase kinase-3β (GSK-3β) inhibitors LiCl or (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO) (Sigma-Aldrich, St. Louis, MO). For comparison, we used nontransfected C57MG cells or C57MG cells transfected with the empty vector pLNCx. The retroviral infection of murine mammary C57MG cells with oncogenic Wnt-1 and the culture of C57MG cells have been described previously [14,15].

For analysis of the influences of GSK-3β inhibition on 24p3 levels, cells were treated for 24 hours with 20 mmol/L LiCl or with 5 μmol/L BIO. Control cells were treated with 20 mmol/L KCl or with 0.2% dimethyl sulfoxide (DMSO). For analysis of the secreted extracellular 24p3 protein, cells were incubated with serum free medium for 24 hours. Protease inhibitors were added to the harvested medium, and the medium was concentrated with an Amicon Ultra-15 filter (Millipore, Billerica, MA).

L-M(TK-) cells (L cells) and Wnt3a expressing L-M(TK-) cells (L Wnt-3a cells) from murine subcutaneous connective tissue were purchased from ATCC. L Wnt-3a cells secrete biologically active Wnt-3a [16]. For preparation of conditioned media from L cells or from L Wnt-3a cells, we followed the ATCC protocol (distributed by LGC Promochem, Wesel, Germany). In each 10-cm dish $2 \times 100{,}000$ cells were seeded and cultured using the recommended medium. At days 4 and 6 after seeding, the media were harvested and pooled, centrifuged at $500 \times g$, and ultrafiltrated. For Wnt pathway activation, the culture medium was replaced by the pooled media from L Wnt-3a cells, or from L cells as control, and the cells were cultured for another 24 hours before analysis. Cell lysates were prepared from cultured cells after detaching from the culture plate with trypsin and resuspending in PBS with protease inhibitors (Roche Diagnostics, Mannheim, Germany). The cell suspension was sonicated and centrifuged at $15{,}000 \times g$ for 15 min at 4°C.

2.2. Northern blot and Western blot analysis

Northern blot analysis was performed to analyze the level of the *24p3* transcript. Denaturing RNA electrophoresis, blotting, and detection of membrane-immobilized RNA fragments by hybridization with a radioactively labeled probe was done according to standard protocols and as previously described [8,17]. The relative levels of proteins in cell lysates or in concentrated culture medium were detected by Western blotting. Protein concentrations in the cell lysate were determined by Bradford assays. Samples were calibrated to equal protein concentrations. After electrophoresis on sodium dodecyl sulfate polyacrylamide gels, gels were electroblotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ). Membranes were probed with primary antibodies against 24p3 [18], β-catenin (BD Transduction Laboratories, BD Biosciences, San Jose, CA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam). Bound antibodies were detected by incubation with secondary horseradish peroxidase (HRP)–coupled donkey–anti-rabbit or secondary HRP-coupled sheep–anti-mouse antibodies and by enhanced chemoluminescence (Amersham Biosciences).

2.3. Real-time quantitative polymerase chain reaction

C57MG cells were cultured in the presence of 5 μmol/L BIO, 800 μmol/L bathophenanthroline disulfonic acid (BPS) (Sigma-Aldrich) or 50 or 100 μmol/L ferric ammonium citrate (FAC) (Sigma-Aldrich). Total RNA was purified with a commercial kit including on-column DNA digestion with RNase-free DNase (Qiagen, Valencia, CA). The RNA was reverse transcribed using oligo-dT as a primer and Omniscript (Qiagen) reverse transcriptase. For real-time quantitative polymerase chain reaction (RT-qPCR), the QuantiTect SYBR Green PCR kit (Qiagen) was used. Fold induction was obtained by using the double delta cycle of threshold (ΔΔCT) method. All samples were referred to the level of the GAPDH transcript. Relative and normalized

units were compared between the samples in the presence of BIO, BPS, or FAC and the controls in the absence of BIO, BPS, or FAC. The following primers were used: GAPDH-fwd: 5′-TGTGTCCGTCGTGGATCTGA-3′, GAPDH-rev: 5′-CCTGCTTCACCACCTTCTTGA-3′, 24p3-fwd: 5′-GGG CAGGTGGTACGTTGTG-3′, 24p3-rev: 5′-TCGTAAAG CTGCCTTCTGTTTTT-3′, TfR-fwd: 5′-TGGCTACCTG GGCTATTGTAA-3′, TfR-rev: 5′-TCTGTCTCCTCCGTT TCAGC-3′.

2.4. Reverse transcriptase PCR (rtPCR)

The presence of the 24p3R transcript in C57MG/Wnt-1 cells and C57MG/pLNCx cells was analyzed by reverse transcriptase PCR (rtPCR). For amplification of 24p3R from mice, the primers 5′-CTTCCTGTTTTATGGCTGGC-3′ and 5′-GGGATGACTTCAGAAGCAAG-3′ were designed based on the two GenBank entries for the murine *24p3R* gene (http://www.ncbi.nlm.nih.gov); these are the cDNA sequences for murine 24p3R, NM_021551 and BC062878 (Fig. 1A). Both sequences originate from the same chromosome by differential splicing. To analyze whether one of the two mRNA species is present in the analyzed cells, we chose the primer sequences according to the following criteria: (i) the primers should anneal to both cDNAs and (ii) the predicted PCR products of the two GenBank entries should differ in length. By this strategy, we would be able to discriminate between the BC062878 and NM_021551 transcripts. The chosen primers annealed on the cDNA sequence NM_021551 from bp 1010 through bp 1030 and from bp 1574 through bp 1594, resulting in a 584-bp rtPCR product. On the cDNA sequence BC062878, the primers annealed from bp 927 through bp 947 and from bp 1648 through bp 1668, resulting in a 741-bp product. The PCR products were cloned by T/A cloning (Invitrogen, Carlsbad, CA) and the inserts were sequenced.

2.5. Gene promoter assay

The gene promoter assay was performed, with marginal modifications, as previously described [17,19]. Cells were grown to 40–70% confluence on 6-cm dishes and then transfected by the calcium phosphate coprecipitation method. We used the following vectors and constructs: pKS+/Ltk80-luc (control vector), topflash-motif in pKS+/Ltk80-luc and the β-galactosidase expression vector pEQ176. The total amount of the transfected DNA was adjusted to 10 μg with the empty vector pBSSK+. For determination of the basal reporter activity, the vectors pCMV and pKS+/Ltk80-luc were transfected. For direct comparison, the topflash-motif in pKS+/Ltk80-luc was used. The topflash-motif is a β-catenin/TCF-responsive promoter consisting of the repetitive TCF-binding site (A/T)(A/T)CAA(A/T)G [19]. We cloned a synthetic oligonucleotide spanning four consecutive motifs in the pKS+/Ltk80-luc vector [19]. For lithium stimulation experiments, cells were incubated with 20 mmol/L LiCl for 24 hours. Cells were harvested and lysed 36 hours after transfection. Luciferase and β-galactosidase activities were determined, and relative luciferase activity was obtained by normalizing luminescence to β-galactosidase activity. In SW480 cells, the Wnt pathway is constitutively activated by mutation and loss of the functional *APC* gene [20]. The Wnt pathway was inhibited by the transfection of wild-type APC (APCwt) in SW480 cells. All gene promoter assays were done in triplicate.

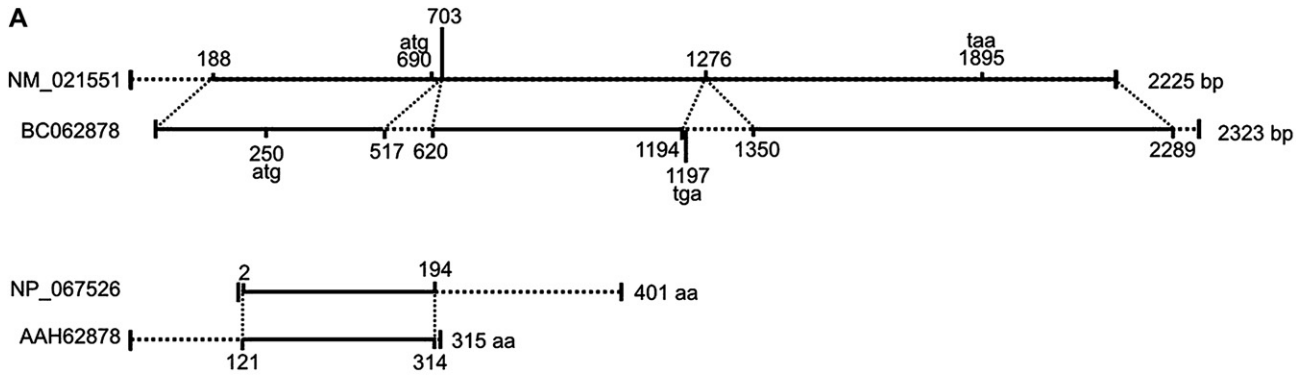
2.6. Determination of intracellular iron and analysis of effects of iron deprivation

Iron concentration was determined in cell lysates by inductively coupled plasma mass spectrometry (ICP-MS) as previously described [21]. Briefly, total cell lysates were adjusted to 0.125% NHOH, 0.005% Triton X100, and 0.05% Titriplex III. The total intensities of all analyzed elements were calibrated to rhodium, which was added as the internal standard. The results were referred to the total amount of intracellular protein. Before the analysis of the 24p3 amount in the absence of iron, C57MG cells were incubated with 800 μmol/L BPS for 24 hours prior to analysis. The reversion of the BPS effect was tested after incubation in 800 μmol/L BPS for 24 hours and addition of FAC at different concentrations for another 24 hours.

3. Results

24p3 is a target gene of the Wnt pathway [8]. We analyzed the intracellular 24p3 protein before and after activation of the Wnt pathway (Fig. 2A). Activation by Wnt-1 transfection or by GSK-3β inhibition led to decrease of the 24p3 protein level. We also identified the extracellular 24p3 protein in the culture medium [18]. The level of extracellular 24p3 decreased after activation of the Wnt pathway. The reduced protein pools of intra- and extracellular 24p3 correlated to the downregulation of the 24p3 transcript as shown by Northern blotting and by qPCR (Fig. 2B).

The 793-bp murine 24p3 promoter (GenBank X81627) includes a TATA box, two overlapping glucocorticoid responsive elements (GREs), and binding sites for the transcription factors Sp1, PEA3, and LF-A1 [22]. We screened the promoter sequence using online binding-site screening programs (SIGSCAN version 4.05, available at http://bimas.dcrt.nih.gov/molbio/signal, and ElDorado version 4.2, available at http://www.genomatix.de), as well as in-house software. The promoter does not comprise any optimal TCF/LEF binding motif 5′-(A/T)(A/T)CAA(A/T)G. Remarkably, the promoter includes neither a metal response nor a stress response element, as one might have concluded from the role of 24p3 in iron transport. We cloned the murine 24p3 promoter from the genomic DNA of C57MG cells and tested whether the 24p3 promoter is regulated by the Wnt pathway by the use of an established



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                1                                                                                                   100
BC062878  (914) CTCCCTGCATCCTCTTCCTGTTTTATGGCTGGCCTGGTCTGTTTCTGGAGTCTGCACGGTGGCTGATAGTGAAGCGGCAGATTGAGGAAGCCCAGTCTGT
  pLNCx     (1) ------------CTTCCTGTTTTATGGCTGGCCTGGTCTGTTTCTGGAGTCTGCACGGTGGCTGATAGTGAAGCGGCAGATTGAGGAAGCCCAGTCTGT
NM_021551 (997) CTCCCTGCATCCTCTTCCTGTTTTATGGCTGGCCTGGTCTGTTTCTGGAGTCTGCACGGTGGCTGATAGTGAAGCGGCAGATTGAGGAAGCCCAGTCTGT
  Wnt-1     (1) ------------CTTCCTGTTTTATGGCTGGCCTGGTCTGTTTCTGGAGTCTGCACGGTGGCTGATAGTGAAGCGGCAGATTGAGGAAGCCCAGTCTGT
                101                                                                                                 200
BC062878 (1014) GCTGAGGATCCTGGCTGAGCGAAACCGGCCCCATGGGCAGATGCTGGGAGAAGAGGCCCAGGAAGCTTTGCAGGAACTGGAGAATACCTGTCCTCTCCCT
  pLNCx    (88) GCTGAGGATCCTGGCTGAGCGAAACCGGCCCCATGGGCAGATGCTGGGAGAAGAGGCCCAGGAAGCTTTGCAGGAACTGGAGAATACCTGTCCTCTCCCT
NM_021551 (1097) GCTGAGGATCCTGGCTGAGCGAAACCGGCCCCATGGGCAGATGCTGGGAGAAGAGGCCCAGGAAGCTTTGCAGGAACTGGAGAATACCTGTCCTCTCCCT
  Wnt-1    (88) GCTGAGGATCCTGGCTGAGCGAAACCGGCCCCATGGGCAGATGCTGGGAGAAGAGGCCCAGGAAGCTTTGCAGGAACTGGAGAATACCTGTCCTCTCCCT
                201                                                                                                 300
BC062878 (1114) GCAACGTCCACCTTTTCCTTTGCGTCCCTCCTCAACTACCGCAACATCTGGAAAAATCTGCTTATCCTGGGCTTCACCAAGTGAGCCTGGCTGTCTGAGC
  pLNCx    (188) GCAACGTCCACCTTTTCCTTTGCGTCCCTCCTCAACTACCGCAACATCTGGAAAAATCTGCTTATCCTGGGCTTCACCAAGTGAGCCTGGCTGTCTGAGC
NM_021551 (1197) GCAACGTCCACCTTTTCCTTTGCGTCCCTCCTCAACTACCGCAACATCTGGAAAAATCTGCTTATCCTGGGCTTCACCAA----------------------
  Wnt-1    (188) GCAACGTCCACCTTTTCCTTTGCGTCCCTCCTCAACTACCGCAACATCTGGAAAAATCTGCTTATCCTGGGCTTCACCAA----------------------
                301                                                                                                 400
BC062878 (1214) TGAGGGCCAGGCCAGTAGCTGGGATAGGGGATGGTGGGGTGGGGGGACCCTCTTGAAGCCCTGCAGAGGATGGAGCTGTGGGAAGCTGTGGATTGGGGTT
  pLNCx    (288) TGAGGGCCAG----------------------------------------------------------------------------------------
NM_021551 (1277) ----------------------------------------------------------------------------------------------------
  Wnt-1    (268) ----------------------------------------------------------------------------------------------------
                401                                                                                                 500
BC062878 (1314) CAGACGCTGTTCTTTGCTTCCCCCACCTGCTGTGCAGCTTTATTGCCCATGCCATTCGCCACTGCTACCAGCCTGTGGGAGGAGGAGGCAGCCCATCAGA
  pLNCx    (298) ----------------------------------------------------------------------------------------------------
NM_021551 (1277) ------------------------------------CTTTATTGCCCATGCCATTCGCCACTGCTACCAGCCTGTGGGAGGAGGAGGCAGCCCATCAGA
  Wnt-1    (268) ------------------------------------CTTTATTGCCCATGCCATTCGCCACTGCTACCAGCCTGTGGGAGGAGGAGGCAGCCCATCAGA
                501                                                                                                 600
BC062878 (1414) CTTCTACTTGTGCTCTCTGCTGGCCAGTGGCACAGCCGCCCTGGCCTGTGTCTTCCTGGGGGTGACTGTGGACCGTTTTGGCCGTCGGGGCATCCTGCTT
  pLNCx    (298) ----------------------------------------------------------------------------------------------------
NM_021551 (1340) CTTCTACTTGTGCTCTCTGCTGGCCAGTGGCACAGCCGCCCTGGCCTGTGTCTTCCTGGGGGTGACTGTGGACCGTTTTGGCCGTCGGGGCATCCTGCTT
  Wnt-1    (331) CTTCTACTTGTGCTCTCTGCTGGCCAGTGGCACAGCCGCCCTGGCCTGTGTCTTCCTGGGGGTGACTGTGGACCGTTTTGGCCGTCGGGGCATCCTGCTT
                601                                                                                                 700
BC062878 (1514) CTCTCAATGACTCTCACGGGGATTGCATCCCTGGTCCTGCTGGGGCTGTGGGATTATCTGAACGATGCTGCCATCACAACCTTCTCTGTCCTTGGCCTCT
  pLNCx    (298) ----------------------------------------------------------ATCTGAACGATGCTGCCATCACAACCTTCTCTGTCCTTGGCCTCT
NM_021551 (1440) CTCTCAATGACTCTCACGGGGATTGCATCCCTGGTCCTGCTGGGGCTGTGGGATTATCTGAACGATGCTGCCATCACAACCTTCTCTGTCCTTGGCCTCT
  Wnt-1    (431) CTCTCAATGACTCTCACGGGGATTGCATCCCTGGTCCTGCTGGGGCTGTGGGATTATCTGAACGATGCTGCCATCACAACCTTCTCTGTCCTTGGCCTCT
                701                                                                                                 800
BC062878 (1614) TCTCCTCCCAAGCTTCTGCTATCCTCAGTACCCTCCTTGCTTCTGAAGTCATCCCCCACCACTGTCCGCGGCCGTGGCCTGGGCCTTATCATGGCACTTGG
  pLNCx    (343) TCTCCTCCCAAGCTTCTGCTATCCTCAGTACCCTCCTTGCTTCTGAAGTCATCCC----------------------------------------------
NM_021551 (1540) TCTCCTCCCAAGCTTCTGCTATCCTCAGTACCCTCCTTGCTTCTGAAGTCATCCCCCACCACTGTCCGCGGCCGTGGCCTGGGCCTTATCATGGCACTTGG
  Wnt-1    (531) TCTCCTCCCAAGCTTCTGCTATCCTCAGTACCCTCCTTGCTTCTGAAGTCATCCC----------------------------------------------
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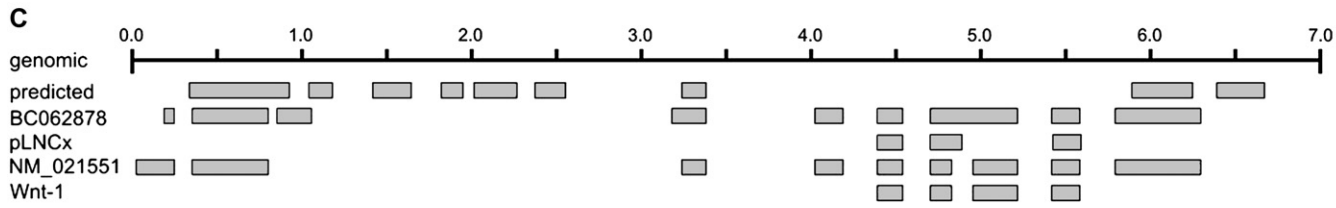

Fig. 1. The two 24p3R cDNAs from GenBank and the amplified 24p3R transcript fragments from C57MG/pLNCx cells and from C57MG/Wnt-1 cells. (A) Schematic presentation of the homologies between the cDNAs NM_021551 and BC062878 and between the predicted protein sequences. Solid lines indicate identical sequence stretches. Dotted lines indicate nonidentical sequence stretches. (B) Alignment of the sequences BC062878 and NM_021551 and the two 24p3R transcripts found in C57MG/pLNCx (pLNCx) and C57MG/Wnt-1 (Wnt-1). (C) Schematic presentation of the 24p3R exons (horizontal gray boxes) on the genomic level. Exons were predicted using GenomeScan software (http://genes.mit.edu/genomescan.html). The cDNAs NM_021551 and BC062878 as well as the 24p3R transcripts amplified from C57MG/Wnt-1 (Wnt-1) or C57MG/pLNCx (pLNCx) cells were blasted. The exons stretch over ~7 kb starting at position 28,282,000 (0.0) of chromosome 14.

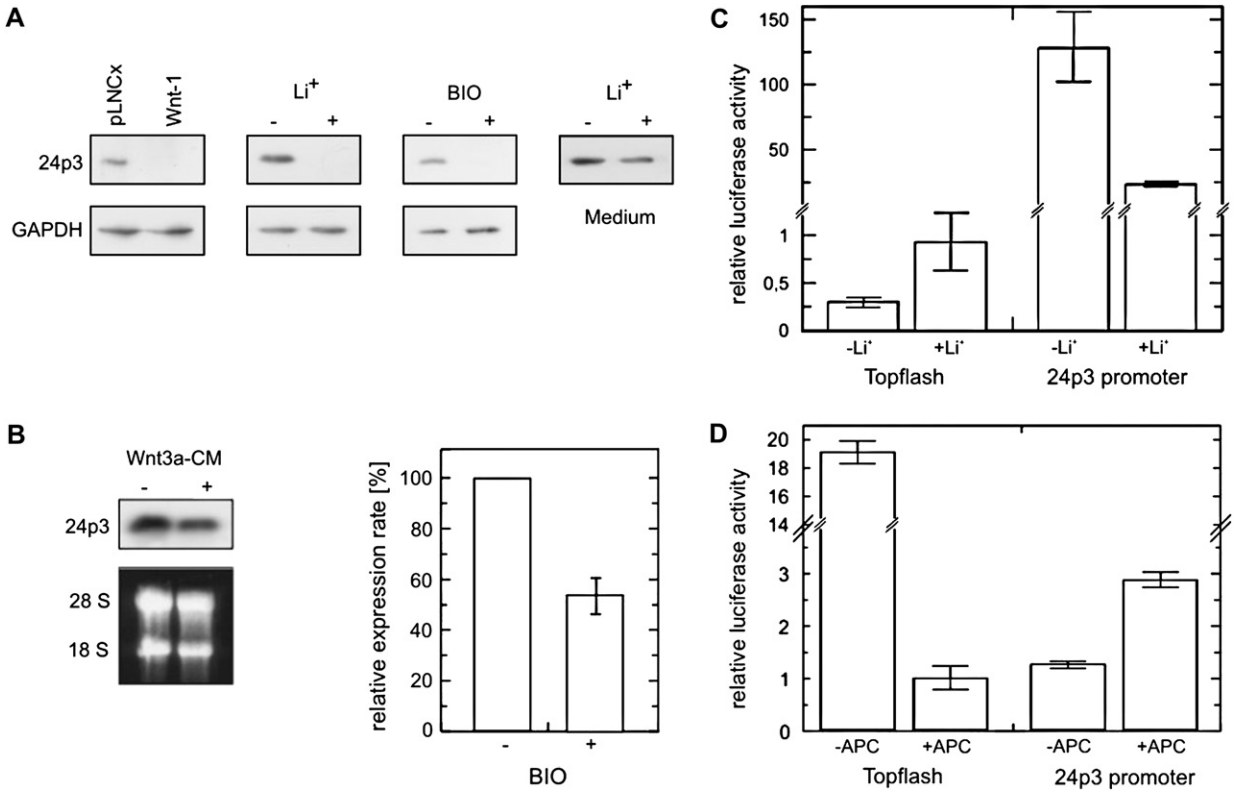

Fig. 2. The relative levels of 24p3 protein and transcript and 24p3 promoter activity in cells with activated Wnt pathway. All cell lysates or culture medium aliquots were equilibrated to equal protein concentrations prior to Western blot analysis. The Western blot analysis of GAPDH is shown as loading control. (A) Western blot analysis of 24p3 in lysates from C57MG/pLNCx (pLNCx) or from C57MG/Wnt-1 (Wnt-1) cells, in lysates from C57MG cells in the absence (−) or presence (+) of LiCl (Li+), in the absence (−) or presence (+) of BIO [(2′Z, 3′E)-6-bromoindirubin-3′-oxime], and in the culture medium of C57MG cells in the absence (−) or presence (+) of LiCl (Li+). (B) Northern blot and real-time quantitative polymerase chain reaction analysis of the 24p3 transcript level in C57MG cells in the absence (−) or presence (+) of conditioned medium from Wnt3a expressing cells (Wnt3a-CM), or in the absence (−) or presence (+) of BIO. (C) The relative activity of the luciferase reporter gene under the control of three Topflash motif was compared with its activity under control of the full-length murine 24p3 promoter. The relative luciferase activity regulated by the Topflash motif or the 24p3 promoter in the absence (−) or presence (+) of LiCl (Li+) in RK13 cells. (D) The relative luciferase activity regulated by the Topflash motif or the 24p3 promoter after transfection with an empty control vector (−APC) or with the full length APCwt cDNA (+APC) in SW480 cells without functional APC. Error bars show standard deviation.

gene promoter assay (Figs. 2C and 2D). The activation of the Wnt pathway by lithium led to the inhibition of the 24p3 promoter. Consistently, the transfection of the APC cDNA into APC-negative SW480 cells, which is equivalent to an inhibition of the Wnt pathway, resulted in the activation of the 24p3 promoter.

The exact regulation of the iron level by intra- and extracellular transport is fundamental for any living cell. Alterations in iron level can lead to G1/S arrest and apoptosis (see Le and Richardson [23] for a review). Iron-free 24p3 lowers intracellular iron levels and induces apoptosis [5]. We determined the relative iron content by means of ICP-MS in cells with different levels of intracellular 24p3 (data not shown). Total cell lysates contained between 38 and 53 ng iron per mg total protein. There was no correlation between iron concentrations and the levels of 24p3. The activation of the Wnt pathway by lithium, by Wnt-3a conditioned medium or by Wnt-1 transfection had no significant influence on the iron level.

Next, we incubated C57MG cells with the iron chelator BPS to test whether the withdrawal of iron had any influence on the 24p3 levels. When iron ions were chelated by BPS, the levels of 24p3 protein and 24p3 transcript decreased significantly (Fig. 3). In contrast to the *24p3* gene, the TfRC RNA increased in the absence of iron. The deprivation of iron had no effect on the level of β-catenin, which is an indicator for Wnt pathway activity. Thus, iron-dependent 24p3 downregulation was independent of the Wnt pathway. The addition of FAC (ferric ammonium citrate) reversed the effect of BPS. These data showed that the Wnt pathway downregulated 24p3 expression, but had no influence on the intracellular iron level. In addition, the extracellular iron level regulated the level of 24p3, but had no effect on the Wnt pathway.

To correlate these findings with 24p3 function, we tested for the presence of 24p3R in the analyzed cell lines and examined whether the activation of the Wnt pathway had an influence on 24p3R transcription. The 24p3R transcripts were amplified by rtPCR from C57MG/Wnt-1 and from C57MG/pLNCx cells. The PCR products from the two cell lines differed in length (Fig. 1B). The 397-bp transcript fragment from C57MG/pLNCx cells was completely

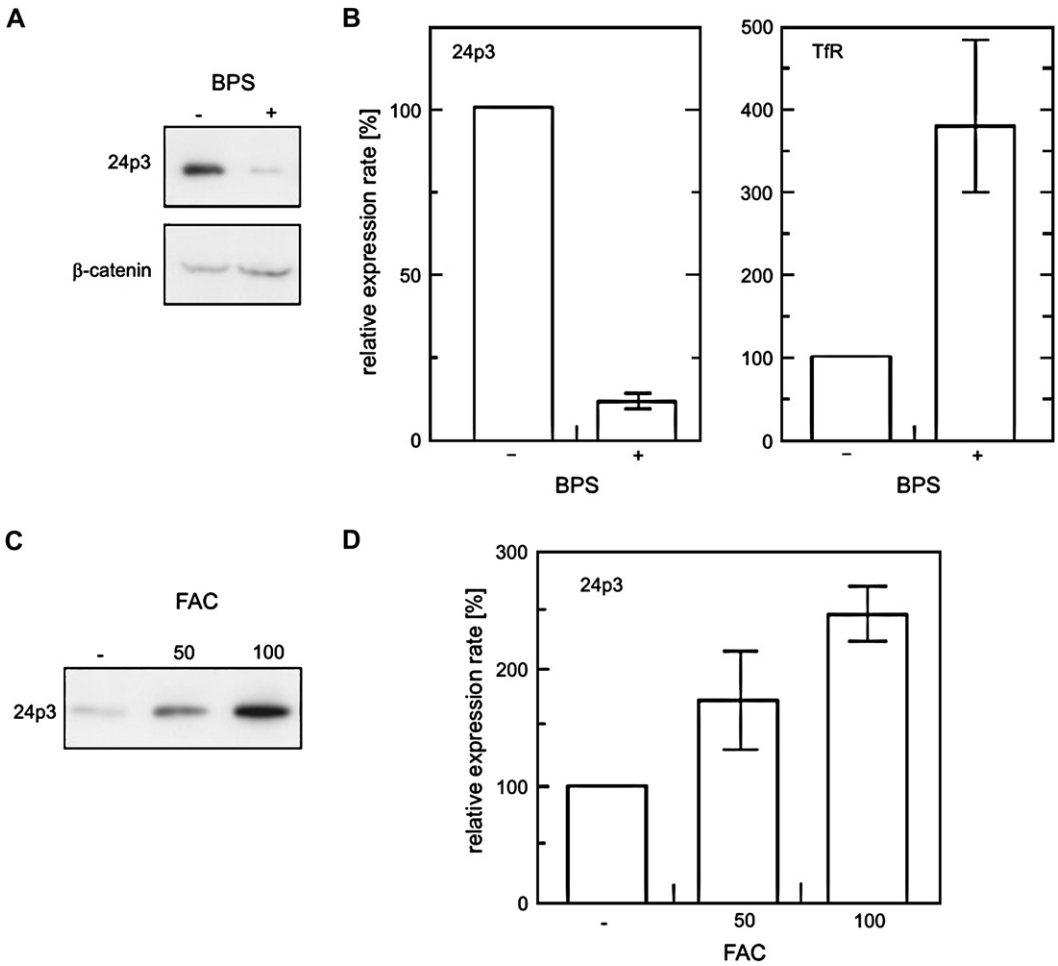

Fig. 3. The influence of the iron level on 24p3. (A) Western blot analysis of 24p3 and β-catenin in the absence (−) or presence (+) of the iron chelating reagent bathophenanthroline disulfonic acid (BPS). (B) Real-time quantitative polymerase chain reaction (qPCR) analysis of the relative expression rate of the genes for 24p3 and for transferrin receptor-1 (TfR) in the absence (−) or presence (+) of BPS. (C) Western blot analysis of the relative 24p3 protein levels in BPS-treated cells in the absence (−) or presence (+) of 50 or 100 μmol/L ferric ammonium citrate (FAC). (D) qPCR analysis of the relative expression rate of the *24p3* gene in BPS-treated cells in the absence (−) or presence (+) of 50 or 100 μmol/L FAC. Error bars show standard deviation.

homologous to parts of the BC062878 sequence. The overlapping region was interrupted by a 344-bp stretch on BC062878. The blast of the C57MG/pLNCx transcript against NM_021551 showed a 364-bp overlap, which was interrupted both on the transcript itself and on NM_021551. The transcript from C57MG/Wnt-1 cells showed the expected length of 584 bp and overlapped completely with the corresponding sequence region of NM_021551. Thus, the 24p3R transcripts differed depending on the Wnt pathway activity: In nonactivated cells the 24p3R gene was transcribed as an as yet unidentified transcript homologous to partial stretches of BC062878. In Wnt-activated cells, the gene was transcribed as a transcript homologous to NM_021551.

4. Discussion

In the present study, we analyzed the lipocalin 24p3 and its regulation by iron and by the Wnt pathway. We found decreased levels of 24p3 protein and transcript in cells with activated Wnt pathway. This decrease occurred under the influence of different activators of the Wnt pathway.

The 24p3 downregulation and the absence of a TCF binding motif in the 24p3 promoter sequence indicated that 24p3 is a secondary or indirect Wnt target. Indirect targets are regulated by one or more primary targets. The Wnt pathway might downregulate 24p3 via a similar mechanism as it downregulates the secondary negative target p21WAF [24]. The cell cycle kinase inhibitor p21WAF is repressed by the direct Wnt target MYC [25]. Two mechanisms of p21WAF repression by MYC are discussed. First, p21Waf might be regulated by the relative MYC levels and the MYC interacting zinc finger protein-1 (MIZ-1) [26]. Secondly, MYC-dependent repression of the p21WAF promoter might be based on interactions between MYC and the zinc finger protein Sp1/Sp3 [27]. The 24p3 promoter includes the two MIZ binding motifs 5′-CCCTGA-3′ and 5′-AAGGAA-3′ and the Sp1 binding sites 5′-GGGCRG-3′ and 5′-GRCGGG-3′. The presence of both MIZ and SP1

binding motifs in the 24p3 promoter sequence supported the possibility that 24p3 is repressed by the direct Wnt target MYC. In analogy to p21WAF, 24p3 might play a role in apoptosis as a negative target of the MYC/MIZ-1 cascade. Thus, 24p3 might be one of the genes, which is downregulated by MYC and by the active Wnt pathway with the effect that apoptosis is inhibited. This model connects our findings with the causal role of 24p3 in apoptosis [5,6].

24p3 can lower the intracellular iron concentration [5]. Wnt pathway induced alterations in the 24p3 level had no effect on the relative intracellular iron content. Although these findings make it unlikely, they did not exclude a potential role of the Wnt regulated 24p3 in iron transport and metabolism. Alterations in the intracellular iron content might be compensated by other proteins, e.g. by regulation of the TfR levels [8].

The deprivation of iron led to the drastic decrease of the 24p3 level. The addition of ionic iron to iron-deprived cells reversed this effect. The cell might react to low extracellular iron levels by downregulation of 24p3 to prevent further iron loss. We conclude that *24p3*, similar to ferritin (*Ftmt*) and TfR (*Tfrc*), is an iron-regulated gene. TfR and ferritin are regulated via an iron-dependent feedback loop on the posttranscriptional level [28]. Transcripts of TfR and ferritin include iron-responsive elements, which affect the stabilities and thus the levels of the transcripts. The 24p3 transcript shows no comparable regulating elements, suggesting a different mechanism of regulation.

The function of 24p3 depends on the iron content of the protein and the expression of its receptor, 24p3R. The differential expression or splicing of 24p3R in Wnt-activated cells might influence the function of 24p3. The lengths of the transcripts identified in Wnt-activated and nonactivated cells differed significantly. C57MG/Wnt-1 cells expressed a transcript, which was identical to the cDNA NM_021551. The complete cDNAs NM_021551 and BC062878 overlap either with exons based on constitutive donor and acceptor sites (GenomeScan; http://genes.mit.edu/genomescan.html) (Fig. 1C) or with exons based on alternative isoforms and cryptic acceptor sites (ASSP alternative splice site predictor; http://es.embnet.org/~mwang/assp.html [29]) (data not shown). The transcript from C57MG/pLNCx cells did not overlap with the predicted exons, indicating a preliminary or a nontranslated transcript. Due to the lack of specific antibodies against a translational product, the search for a new 24p3R protein and a final conclusion about the biological relevance of this transcript were not possible.

In summary, we have described 24p3 as a lipocalin gene that is independently regulated by iron and by the Wnt pathway. The Wnt pathway regulated 24p3R expression on the transcript level and led to expression of alternative spliced forms of the receptor. Thus, the differential expression of the 24p3 gene together with the expression of different 24p3R isoforms might determine the biological role of 24p3 in cells with active Wnt pathway.

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