ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 309 (2003) 52–57

BBRC

www.elsevier.com/locate/ybbrc

Identification and characterization of *Xenopus* NDRG1

Jun-ichi Kyuno,[a] Akimasa Fukui,[a] Tatsuo Michiue,[b] and Makoto Asashima[a,b,*]

[a] *Department of Life Sciences (Biology), Graduate School of Arts and Sciences, University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153-8902, Japan*

[b] *Solution Oriented Research for Science and Technology (SORST) Project, Japan Science and Technology Corporation (JST), Komaba 3-8-1, Meguro-ku, Tokyo 153-8902, Japan*

Received 22 July 2003

Abstract

NDRG1 is a member of the N-myc downstream-regulated gene (NDRG) family and is involved in cellular differentiation, activation of p53, cell cycle arrest, metastasis, and hypoxia. Expression of NDRG1 is repressed by the proto-oncogene, N-myc during mouse development, although the exact functional role of NDRG1 in development remains unknown. Here, we report the characterization of *Xenopus laevis* NDRG1 (xNDRG1) during *X. laevis* development. Expression of xNDRG1 transcript was first detected at stage 15, and was localized to the presumptive pronephric anlagen at stage 26 and to pronephros, eye, branchial arches, and tail-bud at stage 32. Overexpression of xNDRG1 results in a reduced pronephros and disorganized somites. Depletion of xNDRG1, using morpholinos, causes failure of pronephros development. These results suggest that xNDRG1 is required for pronephros development in *X. laevis*.

Keywords: Pronephros; *Xenopus laevis*; Development; N-myc downstream-regulated gene

N-myc downstream-regulated gene 1 (NDRG1) is one of the four members of a new gene family containing no protein motifs with a known function [1,2]. NDRG1 was independently reported with the different names of Ndr1 [3], Drg1 [4], Cap43 [5], rit43 [6], RTP [7], and TDD5 [8]. NDRG1 mRNA and protein levels are modulated under various conditions such as cellular differentiation [4,9,10], activation of p53 [6,11], cell cycle arrest [6,12], metastasis [13], and hypoxia [14–16]. Various chemical agents including synthetic ligands of the PPARγ/RXR transcriptional pathway [12], histone deacetylase and DNA methylation inhibitors [13], nickel compounds [5], and calcium ionophores [5,17] can activate NDRG1 expression in several cell types. Restoration of NDRG1 expression in neoplastic cells results in a growth inhibitory effect [6] and overexpression in metastatic colon cancer cells causes morphological changes [13].

Expression of NDRG1 is repressed by N-myc and c-myc, and up-regulated in N-myc-deficient mouse embryos [3], which show abnormalities in many organs [18–20]. However, a functional role for NDRG1 in development has yet to be defined. We isolated *Xenopus laevis* NDRG1 (xNDRG1) as a candidate gene respondent to vector-averaged gravity (clinostat rotation) in A6 cells derived from the kidney of *X. laevis*. In this study, we aimed to characterize xNDRG1 during development by examining the temporal and spatial expression patterns of xNDRG1 transcripts in embryos and using morpholino oligonucleotides to inhibit the translation of xNDRG1. Although the function of xNDRG1 remains unclear, our results suggest that xNDRG1 is necessary for pronephros development in *X. laevis*.

Materials and methods

Eggs and embryos. Xenopus eggs were obtained by injecting adult males with 300 U of human chorionic gonadotropin (Gestron; Denka Seiyaku) and fertilized in vitro. Staging of embryos was according to

* Corresponding author. Fax: +81-3-5454-4330.
E-mail address: asashi@bio.c.u-tokyo.ac.jp (M. Asashima).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-291X(03)01522-5

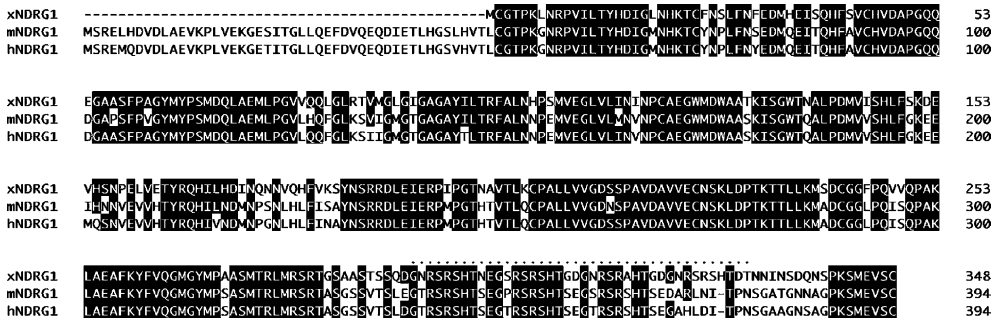

Fig. 1. Alignment of the xNDRG1 amino acid sequence with mouse NDRG1 (mNDRG1) and human NDRG1 (hNDRG1). The xNDRG1 protein has 75.9% identity to hNDRG1 and 75.0% identity to mNDRG1. Black boxes represent identical amino acids. The dotted line indicates a tandem repeat of a homologous sequence comprising 10 amino acids.

Nieuwkoop and Faber [21]. The jelly coat was removed with Steinberg's solution containing 4% cysteine hydrochloride (pH 7.8).

RT-PCR. Five hundred nanograms of total RNA isolated from embryos was used to generate first-strand cDNA. RT-PCR was done in the exponential phase of amplification. Primers used in this study were as follows: xNDRG1 (5′-CTTAATGATGCATGTAGTCTTGC TA-3′ and 5′-TATACATACGCTGTGTTTGC-3′), ornithine decarboxylase (ODC) (5′-GAACAGCTTCAGCAATGACG-3′ and 5′-GG AGAGAGTCTTCACAATGGC-3′).

Whole-mount in situ hybridization. Whole-mount in situ hybridization was carried out as described elsewhere [22]. Digoxigenin (DIG)-labeled RNA probe was synthesized from cDNA corresponding to full-length xNDRG1. The probe was hybridized to a developmental series of whole albino *Xenopus* embryos. The color reaction was performed using BM purple (Roche Diagnostics GmbH, Mannheim, Germany).

Immunohistochemistry and histological examination. For X-gal and Red-gal staining, embryos were fixed in MEMFA for 1 h at 4 °C, washed in phosphate-buffered saline (PBS), and incubated in X-gal or Red-gal buffer (1 mg/ml X-gal or Red-gal, 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$, and 0.1% Tween 20 in PBS) at 37 °C. For immunohistochemistry, samples fixed with MEMFA were washed in PBS and dehydrated in methanol overnight. Embryos were then rehydrated in PBS, blocked with 20% FBS in PBS containing 0.2% BSA and 0.1% Triton X-100, and then incubated in primary antibody overnight at 4 °C. Monoclonal antibodies 3G8 and 4A6 were used as primary antibodies to recognize pronephric tubules and ducts, respectively [23,24]. After washing in PBS, embryos were blocked again and incubated in alkaline phosphatase-conjugated secondary antibody overnight at 4 °C. Finally, the embryos were washed in PBS and stained using NBT/BCIP. For histological examination, embryos were fixed in Bouin's solution for 3 h. They were then dehydrated through a graded series of ethanol, transferred to xylene for embedding in paraffin, and sectioned at 8 μm. Sections were stained with Delafield's hematoxylin and eosin.

Construction of expression vector and microinjections. The open reading frame of xNDRG1 was PCR-amplified using the primers, 5′-CAGAATTCTATGTGTGGGACGCCCCAAGCTG-3′ and 5′-ATG AATTCTAACTTAGCAGGAGACTTCCAT-3′. The PCR product was digested with *Eco*RI and cloned in-frame into pCS2-myc (pCS2-myc-xNDRG1). The pCS2-myc-xNDRG1 construct was linearized by *Not*I and transcribed with the mMESSAGE mMACHINE SP6 Kit (Ambion, Texas, USA). Two nanograms of myc-xNDRG1 mRNA was co-injected with 200 pg of lacZ mRNA, as a lineage tracer, into the lateral marginal zone of 4-cell stage embryos.

Morpholino oligonucleotides. All morpholino oligonucleotides (MOs) were designed and supplied by Gene Tools, LLC (Oregon, USA). MOs used in this study were as follows: xNDRG1-MO (5′-GTTCAGCTTGGGCGTCCCACACATA-3′), Control-MO (5′-CCT CTTACCTCAGTTACAATTTATA-3′).

Results and discussion

Sequence analysis of X. laevis NDRG1

Xenopus laevis N-myc downstream-regulated gene 1 (xNDRG1) was obtained as a clone of 2533 nucleotides, containing a complete open reading frame of 1047 bases, a 5′ UTR of 253 bases and a 3′ UTR of 1233 bases. Conceptual translation of xNDRG1 yielded a predicted protein sequence of 348 amino acid residues, which displays approximately 75% identity with NDRG1 proteins in other vertebrate species. xNDRG1 is unique among the NDRG family in that it lacks 47 amino acids at the N-terminus and has a tandem repeat of a homologous sequence comprising 10 amino acids (Fig. 1).

Temporal and spatial distribution of xNDRG1 during X. laevis development

A functional analysis of NDRG1 in development has yet to be reported despite N-myc being reported to repress NDRG1 expression during mouse ontogenesis [4]. To address this, we analyzed the temporal expression profile of xNDRG1 transcripts by RT-PCR (Fig. 2). Maternal expression of xNDRG1 was not detected. Zygotic expression was first detected at a low level at

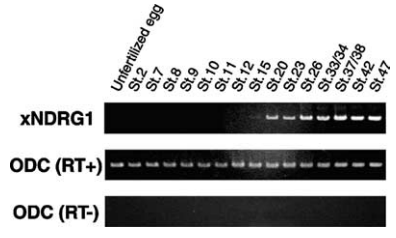

Fig. 2. Temporal expression pattern of xNDRG1 during development detected by RT-PCR. RNA was extracted from embryos at the stage indicated over each lane. RT-PCR using an ornithine decarboxylase (ODC)-specific primer was carried out in parallel to control the amount of input RNA [ODC (RT+)]. RT-PCR without reverse transcriptase showed no contamination of genomic DNA [ODC (RT−)].

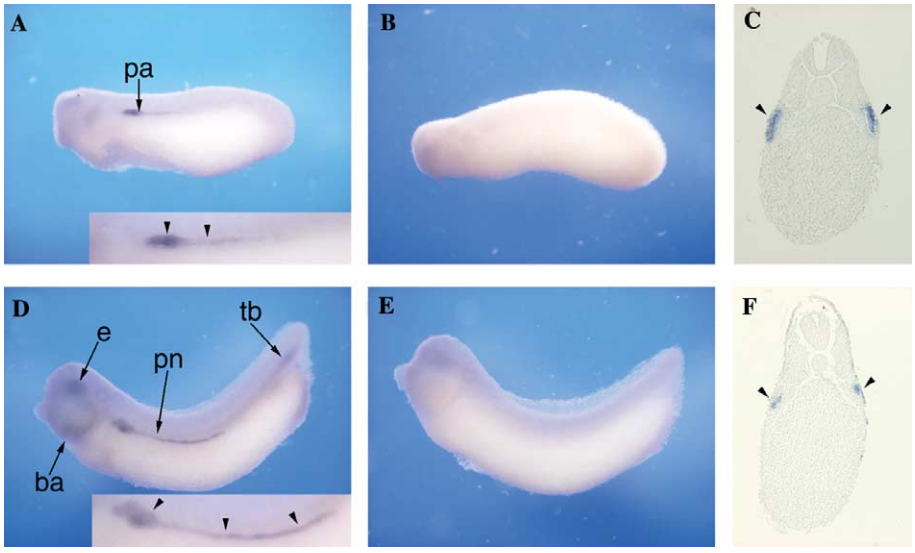

Fig. 3. Spatial expression pattern of xNDRG1 during development detected by whole-mount in situ hybridization. (A,B) Lateral view of a stage-26 embryo hybridized with xNDRG1 antisense (A) and sense probes (B). (C) Transverse section of the embryo in A. (D,E) Lateral view of a stage-32 embryo hybridized with antisense (D) and sense probes (E). (F) Transverse section of the embryo in D. Insets in A and D indicate higher magnifications of the pronephric region. The antisense probe hybridized with the presumptive pronephric anlagen (pa) at stage 26 (arrowheads in A and C). At stage 32, xNDRG1 transcripts were clearly detected in pronephros (pn) (arrowheads in D and F), eye (e), branchial arches (ba), and tail-bud (tb). A sense xNDRG1 control probe showed no staining (B,E).

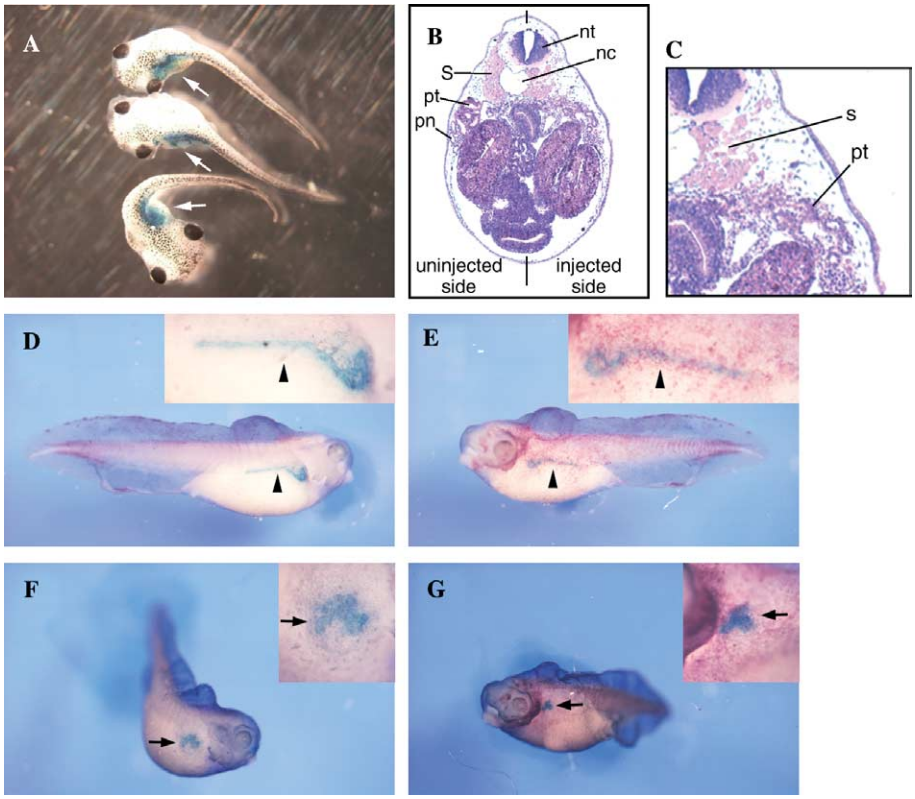

Fig. 4. Overexpression of xNDRG1 by injecting myc-xNDRG1 mRNA. Synthesized myc-xNDRG1 mRNA (2 ng) and lacZ mRNA (200 pg) were hemilaterally co-injected into the lateral marginal zone of 4-cell stage embryos. (A) Embryos stained with X-gal. Blue color shows the localization of co-injected lacZ (white arrow). (B) Section through the pronephros of injected embryos at stage 45. (C) Higher magnification of B. Notchord (nc), neural tube (nt), pronephric tubule (pt), pronephric duct (pn), and somite (s) are indicated. (D,E) Uninjected side (D) and injected side (E) of an embryo stained with pronephric duct-specific antibody 4A6 (arrowhead marks the pronephric ducts). (F,G) Uninjected (F) and injected sides (G) of an embryo stained with pronephric tubule-specific antibody 3G8 (arrow marks the pronephric tubules). Insets in D–G indicate higher magnification images of the pronephric region. Co-injected lacZ mRNA was detected by Red-gal staining only at the injected side (E,G).

stage 15 and markedly increased up to stage 20 after which time the level of expression remained stable.

The spatial expression pattern of xNDRG1 was examined by whole-mount in situ hybridization (Figs. 3A, C, D, and F). The xNDRG1 mRNA was detected in the presumptive pronephric anlagen at stage 26 (Fig. 3A), and in the early tadpole stage (stage 32), xNDRG1 transcripts were predominantly expressed in pronephros, with lower levels of expression detected in eye, branchial arches, and tail-bud (Fig. 3D). The sections through the pronephros showed high levels of expression (arrowheads in Figs. 3C and F). Since specification of the pronephros is established between stages 12.5 and 14 in *X. laevis* embryogenesis [25,26], our temporal and spatial expression analyses suggested that xNDRG1 might play a role in pronephros development after specification of the pronephros.

Overexpression of xNDRG1 leads to pronephric and somatic defects

To investigate the function of xNDRG1 in pronephros development, we examined the effects of overexpression. The myc-xNDRG1 mRNA was injected into the lateral marginal zone of 4-cell stage embryos along with 200 pg of mRNA encoding lacZ as a lineage tracer (Fig. 4). In almost all cases, embryos were deformed at the injected side (Fig. 4A). Pronephros development was examined by immunohistochemical staining of pronephric ducts (Figs. 4D and E) and tubules (Figs. 4F and G). Compared to the uninjected side (Figs. 4D and F), formation of the pronephric ducts on the injected side was disrupted (Fig. 4E), and the size of pronephric tubules was reduced (Fig. 4G). Sections through the pronephros of injected embryos confirmed the disorganized formation of pronephros and somites only at the injected side (Figs. 4B and C).

Our results to this point suggested that appropriate expression of xNDRG1 is required for normal formation of the pronephros. As anterior somites are necessary for pronephros development [27,28], the pronephros of the injected embryos might have been disrupted, indirectly, via disorganization of the anterior somites. However, immunostaining of the pronephric ducts and tubules with specific antibodies indicated the presence of differentiation resulting in normal pronephros basic structures, suggesting that inductive activity of the anterior somites was not inhibited by the overexpression of xNDRG1. Furthermore, NDRG1 can cause morphological changes [14] and cell cycle arrest [7,13] in several cell types. Therefore, gain of xNDRG1 function may affect morphological and/or proliferative events in pronephric and somatic development.

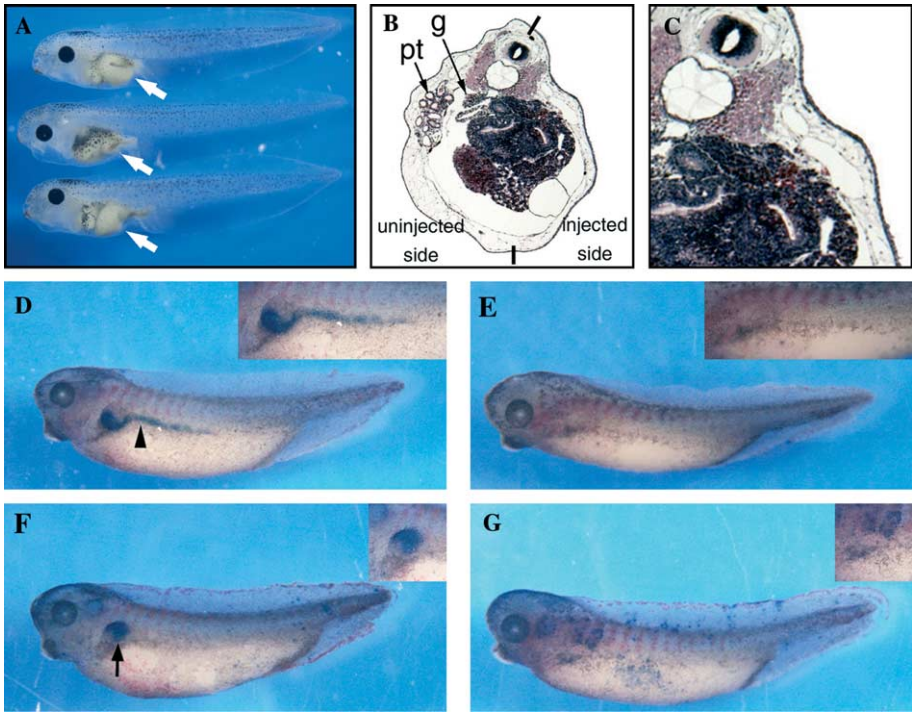

Fig. 5. Depletion of xNDRG1 by injecting xNDRG1-MO. MO (17 ng) and lacZ mRNA (200 pg) were hemilaterally co-injected into the lateral marginal zone of 4-cell stage embryos. (A) xNDRG1-MO-injected embryos at stage 45 show disorganization of gut coiling (white arrow). (B,C) Section through the pronephros of xNDRG1-MO-injected embryos and higher magnification of injected side of B. No pronephric tubule (pt) and glomus (g) are seen at the injected sides. (D–G) Injections of xNDRG1-MO (E,G) or Cont-MO (D,F). Resulting embryos were stained with 4A6 (D,E) and 3G8 (F,G). Arrowhead and arrow marks the pronephric ducts and the pronephric tubules, respectively. Insets in D–G indicate higher magnification images of the pronephric region. Co-injected lacZ mRNA was detected by Red-gal staining.

Translational inhibition of xNDRG1 causes failure of pronephric development

To address whether xNDRG1 gene function is necessary for pronephric development, we performed translational inhibition by injecting antisense morpholino oligonucleotides (MOs). We prepared xNDRG1-MO to target the translational start site (position −1 to +24) of xNDRG1 and standard Cont-MO as a control. Injections of 2 pmol (17 ng) of each MO were made into the lateral marginal zone of 4-cell stage embryos along with 200 pg of mRNA encoding lacZ as a lineage tracer (Fig. 5). In almost all cases, xNDRG1-MO-injected embryos showed disorganization of gut coiling (Fig. 5A). Sections through the pronephros of xNDRG1-MO-injected embryos showed failure of pronephric development on the injected side (Figs. 5B and C). The Cont-MO-injected embryos developed normally. Immunohistochemical staining showed that injection of the xNDRG1-MO efficiently abolished staining of the pronephric ducts and tubules (Figs. 5E and G), with normal staining detected in the Cont-MO-injected embryos (Figs. 5D and F).

The immunostaining of the pronephric ducts and tubules also indicated an absence of pronephric epithelia. In addition, basic structures of the pronephros and pronephric cells were not detectable in the xNDRG1-MO-injected embryos. These results suggest that xNDRG1 is required for differentiation and/or survival of pronephric cells.

Taken together, the results presented here indicate that xNDRG1 function is necessary for normal development of the pronephros and suggest that xNDRG1 is important for the differentiation, survival, proliferation, and morphogenesis of pronephric cells. The mechanisms of these proposed functions remain to be elucidated and further analysis is necessary.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology and by SORST projects of the Japan Science and Technology Corporation. We thank Prof. Naoto Ueno and Dr. Atsushi Kitayama for providing materials. We acknowledge valuable discussion with Dr. Te-chuan Chan.

References

[1] T. Okuda, H. Kondoh, Identification of new genes ndr2 and ndr3 which are related to Ndr1/RTP/Drg1 but show distinct tissue specificity and response to N-myc, Biochem. Biophys. Res. Commun. 266 (1999) 208–215.

[2] R.H. Zhou, K. Kokame, Y. Tsukamoto, C. Yutani, H. Kato, T. Miyata, Characterization of the human NDRG gene family: a newly identified member, NDRG4, is specifically expressed in brain and heart, Genomics 73 (2001) 86–97.

[3] A. Shimono, T. Okuda, H. Kondoh, N-myc-dependent repression of ndr1, a gene identified by direct subtraction of whole mouse embryo cDNAs between wild type and N-myc mutant, Mech. Dev. 83 (1999) 39–52.

[4] N. van Belzen, W.N. Dinjens, M.P. Diesveld, N.A. Groen, A.C. van der Made, Y. Nozawa, R. Vlietstra, J. Trapman, F.T. Bosman, A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms, Lab. Invest. 77 (1997) 85–92.

[5] D. Zhou, K. Salnikow, M. Costa, Cap43, a novel gene specifically induced by Ni^{2+} compounds, Cancer Res. 58 (1998) 2182–2189.

[6] S.K. Kurdistani, P. Arizti, C.L. Reimer, M.M. Sugrue, S.A. Aaronson, S.W. Lee, Inhibition of tumor cell growth by RTP/rit42 and its responsiveness to p53 and DNA damage, Cancer Res. 58 (1998) 4439–4444.

[7] K. Kokame, H. Kato, T. Miyata, Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis: GRP78/BiP and novel genes, J. Biol. Chem. 271 (1996) 29659–29665.

[8] T.M. Lin, C. Chang, Cloning and characterization of TDD5, an androgen target gene that is differentially repressed by testosterone and dihydrotestosterone, Proc. Natl. Acad. Sci. USA 94 (1997) 4988–4993.

[9] B. Xu, L. Lin, N.S. Rote, Identification of a stress-induced protein during human trophoblast differentiation by differential display analysis, Biol. Reprod. 61 (1999) 681–686.

[10] W. Ulrix, J.V. Swinnen, W. Heyns, G. Verhoeven, The differentiation-related gene 1, Drg1, is markedly upregulated by androgens in LNCaP prostatic adenocarcinoma cells, FEBS Lett. 455 (1999) 23–26.

[11] J. Yu, L. Zhang, P.M. Hwang, C. Rago, K.W. Kinzler, B. Vogelstein, Identification and classification of p53-regulated genes, Proc. Natl. Acad. Sci. USA 96 (1999) 14517–14522.

[12] D. Piquemal, D. Joulia, P. Balaguer, A. Basset, J. Marti, T. Commes, Differential expression of the RTP/Drg1/Ndr1 gene product in proliferating and growth arrested cells, Biochim. Biophys. Acta 1450 (1999) 364–373.

[13] R.J. Guan, H.L. Ford, Y. Fu, Y. Li, L.M. Shaw, A.B. Pardee, Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer, Cancer Res. 60 (2000) 749–755.

[14] H. Park, M.A. Adams, P. Lachat, F. Bosman, S.C. Pang, C.H. Graham, Hypoxia induces the expression of a 43-kDa protein (PROXY-1) in normal and malignant cells, Biochem. Biophys. Res. Commun. 276 (2000) 321–328.

[15] K. Salnikow, M. Costa, W.D. Figg, M.V. Blagosklonny, Hyperinducibility of hypoxia-responsive genes without p53/p21-dependent checkpoint in aggressive prostate cancer, Cancer Res. 60 (2000) 5630–5634.

[16] K. Salnikow, W. Su, M.V. Blagosklonny, M. Costa, Carcinogenic metals induce hypoxia-inducible factor-stimulated transcription by reactive oxygen species-independent mechanism, Cancer Res. 60 (2000) 3375–3378.

[17] K. Salnikow, T. Kluz, M. Costa, Role of Ca^{2+} in the regulation of nickel-inducible Cap43 gene expression, Toxicol. Appl. Pharmacol. 160 (1999) 127–132.

[18] S. Sawai, A. Shimono, Y. Wakamatsu, C. Palmes, K. Hanaoka, H. Kondoh, Defects of embryonic organogenesis resulting from targeted disruption of the N-myc gene in the mouse, Development 117 (1993) 1445–1455.

[19] C.B. Moens, B.R. Stanton, L.F. Parada, J. Rossant, Defects in heart and lung development in compound heterozygotes for two different targeted mutations at the N-myc locus, Development 119 (1993) 485–499.

[20] C.M. Bates, S. Kharzai, T. Erwin, J. Rossant, L.F. Parada, Role of N-myc in the developing mouse kidney, Dev. Biol. 222 (2000) 317–325.
[21] P.D. Nieuwkoop, J. Faber, Normal Table of *Xenopus laevis* (Daudin), Elsevier, North-Holland, Amsterdam, 1967.
[22] R.M. Harland, In situ hybridization: an improved whole-mount method for *Xenopus* embryos, Methods Cell Biol. 36 (1991) 685–695.
[23] P.D. Vize, E.A. Jones, R. Pfister, Development of the *Xenopus* pronephric system, Dev. Biol. 171 (1995) 531–540.
[24] T.C. Chan, S. Takahashi, M. Asashima, A role for *Xlim-1* in pronephros development in *Xenopus laevis*, Dev. Biol. 228 (2000) 256–269.
[25] H.C. Brennan, S. Nijjar, E.A. Jones, The specification and growth factor inducibility of the pronephric glomus in *Xenopus laevis*, Development 126 (1999) 5847–5856.
[26] H.C. Brennan, S. Nijjar, E.A. Jones, The specification of the pronephric tubules and duct in *Xenopus laevis*, Mech. Dev. 75 (1998) 127–137.
[27] D.W. Seufert, H.C. Brennan, J. DeGuire, E.A. Jones, P.D. Vize, Developmental basis of pronephric defects in *Xenopus* body plan phenotypes, Dev. Biol. 215 (1999) 233–242.
[28] T.J. Mauch, G. Yang, M. Wright, D. Smith, G.C. Schoenwolf, Signals from trunk paraxial mesoderm induce pronephros formation in chick intermediate mesoderm, Dev. Biol. 220 (2000) 62–75.