From the Institute of Physiological Chemistry, Medical Faculty of the Martin-Luther-University of Halle-Wittenberg

(Director: Prof. Dr. Guido Posern)

Identification of new target genes of the transcriptional regulator Ets-related protein 71

Dissertation
to obtain the academic degree
Doctor rerum medicarum (Dr. rer. medic.)
for the field of Medical Physiology and Pathophysiology

submitted to
the Medical faculty
of the Martin-Luther-University Halle-Wittenberg

by Aisha Ayad Argai
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Aus dem Institut für Physiologische Chemie der Medizinischen Fakultät der Martin-Luther-Universität Halle-Wittenberg

(Direktor: Prof. Dr. Guido Posern)

Identifizierung neuer Zielgene des transkriptionallen Regulators Ets-related protein 71

Dissertation
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vorgelegt
der Medizinischen Fakultät
der Martin-Luther-Universität Halle-Wittenberg

von Aisha Ayad Argai
geboren am 29. Mai 1978 in Shahat (Libya)

Betreuer: Prof. Dr. Thomas Hollemann

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Öffentliche Verteidigung am 16.1.2015

To my children

Amena, Anas and Esra

Referat

Vaskulogenese und die Bildung des Gefäßsystems sind essentielle Schritte während der Embryonalentwicklung. Kürzlich wurde ER71 als Mitglied der Familie der ETS-Transkriptionsfaktoren identifiziert, welches wesentlich an der Regulation der Gefäßbildung beteiligt ist. Da die Überexpression von ER71 zu Gastrulationsdefekten führt, war es schwierig die Funktion von ER71 durch Überexpression im gesamten Embryo zu analysieren. Daher stellte ich ein Plasmidkonstrukt (ER71-GR) zur Expression eines Dexamethason-induzierbaren ER71-Proteins her. Dieses Konstrukt machte es möglich den Effekt der Überexpression von ER71 auch während späterer Entwicklungsstadien von *Xenopus laevis* zu analysieren. Damit konnte ich zeigen, dass zwei bekannte Zielgene von ER71, nämlich *ami* und *lmo2*, während unterschiedlicher Entwicklungsphasen durch ER71 regulierbar waren und dass die Anzahl der Zellen, die auf die Überexpression von ER71 reagieren, zwischen NF St. 10 und NF St. 20 deutlich zunahm. Das Hauptziel dieser Dissertation war die Identifizierung neuer Zielgene von ER71. Daher nutzte ich das ER71-GR-Konstrukt zur Überexpression von ER71 in animalen Kappen. Die anschließende Behandlung mit Dexamethason führte reproduzierbar zur Induktion bekannter ER71-Zielgene. Zur Identifizierung neuer Zielgene kollaborierten wir mit der Arbeitsgruppe von Thomas Pieler in Göttingen und nutzten die dort etablierte ‚RNA deep sequencing' Technologie. Damit konnten wir eine ganze Liste von Genen identifizieren, deren Expression in Dexamethason behandelten ER71 überexprimierenden animalen Kappen hochreguliert war. Diese Liste beinhaltet bekannte ER71-Zielgene, was beweist, dass die Dexamethasonbehandlung von Er71 überexprimierenden animalen Kappen, zur Transkription von ER71-Zielgenen führte. Weiterhin konnten ER71-Zielgene identifiziert werden, von welchen bereits bekannt war, dass sie eine Rolle während der Vaskulogenese und Hämatopoese spielen. Das interessanteste Ergebnis war die Identifizierung neuer potentieller ER71-Zielgene (*Cplx2*, *march2*, *rgl2* und *zeb2*), deren Einfluss auf die Vaskulogenese bisher nicht gezeigt wurde. In unabhängigen Experimenten konnte ich die Induktion der Transkription von mehr als 50% der identifizierten ER71-Zielgene bestätigen.

Zusammenfassend lässt sich feststellen, dass es mit dieser Arbeit gelungen ist eine große Anzahl unbekannter ER71-Zielgene zu identifizieren, deren genaue Funktion während der Entwicklung eines funktionierenden Gefäßsystems nun in weiteren Analysen untersucht werden kann.

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Table of Contents

Table of Contents

Table of Contents

List of Figures

List of Tables

Abbreviations

AP	Alkaline phosphatase
Ang1	Angiopoietin-1
APB	Alkaline phosphatase buffer
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BMB	Boehringer blocking reagent
BNIP3	Bcl2/EIB 19kDa-interacting protein 3
BSA	Bovine Serum Albumin
ºC	Degree centigrade
CAD	C-terminal transactivation domain
CDKN	Cyclin-dependent kinase inhibitor gene
cDNA	Complementary DNA
DAPI	4',6'-Diamidin-2'-phenylindol-dihydrochloride
DA	Dorsal aorta
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
Dex	Dexamethasone
Dig	Digoxigenin-11-2'-deoxuridin-5'-triphosphat
DLAV	Dorsal longitudinal anastomosing vessel
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethlene glcycol-bis (2-amino-ethylether-N,N,N',N')-tetra-acetic acid
ER71	Ets-related protein 71
et al.	*et alii*
FGF-2	Fibroblast derived growth factor-2
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
HCG	Human chorionic gonadotropin
Hpf	Hours past fertilization
Hrs	Hours
HS	Horse serum

Abbreviations

HUVEC	Human umbilical vein endothelial cell
ISV	Intersomitic veins
kb	Kilobase
kDa	Kilo Dalton
M	Molar (mol/l)
MAB	Maleic acid buffer
MBS	Modified Barth's Saline
MEM	MOPS/EGTA/Magnesium sulfate buffer
MEMFA	MOPS/EGTA/Magnesium sulfate/formaldehyde buffer
MO	Morpholino Oligonucleotides
MOPS	4-morpholinpropanosulfonic acid
NBT	Nitro blue tetrazolium chloride
NF Stage	Nieuwkoop and Faber stage
ODD	Oxygen-dependent degradation domain
ORF	Open Reading Frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCV	Posterior cardinal vein
PDGF	Platelet derived growth factor
Pg	Pico gram
pH	Potential hydrogen
TE buffer	Tris/EDTA buffer
TGFβ	Tumour growth factor β
UTR	Un-translated region
U	Unit
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
w/v	Weight in volume

1. Introduction

1.1 General Introduction to Embryonic Vasculogenesis

During embryogenesis, the development of the vascular system is required to provide oxygen and nutrients to the growing tissue. The vascular system is made up of a complex network of veins, arteries, and capillaries. These vessels are hollow tubes that must be stiff enough to withstand the pressure of blood flow, and elastic enough to compensate changes in blood pressure. The development of the vascular system begins with the specification of endothelial precursor cells from the mesoderm. Blood vessels and blood cells share the same precursor cells, which are called hemangioblasts and which give rise to pluripotent hematopoietic stem cells and angioblasts. Pluripotent hematopoietic stem cells differentiate into lymphocyte stem cells and blood stem cells. Numerous types of blood cells are formed from the blood stem cell. On the other hand, angioblasts give rise to endothelial cells, which form the lining of blood vessels (Amatruda and Zon, 1999). The process by which angioblasts are first specified and assembled into primary blood vessels is called vasculogenesis. All subsequent growth and elaboration of the vascular network occurs by branching from preexisting vessels. This process is called angiogenesis. Angiogenesis is a normal process that occurs in development of the growing embryo, as well as in wound healing. However, it is also an essential step in tumor progression and metastasis in cancer.

Various growth factors are responsible for the formation of the vascular system, such as basic fibroblast growth factor (FGF2) (Ribatti et al., 1995), vascular endothelial growth factor (VEGF) (Millauer et al., 1993), angiopoietin-1 (Ang1) (Davis et al., 1996; Suri et al., 1996), platelet derived growth factor (PDGF) and tumour growth factor β (TGFβ) (Lindahl et al., 1997). Vasculogenesis is started by FGF2 signalling. FGF2 ligand binds to FGF receptor and initiates the differentiation of mesodermal cells into hemangioblasts. These cells aggregate and form blood islands. The inner cells in these blood islands become hematopoietic stem cells and the outer cells become angioblasts (Hanahan, 1997). VEGF supports the differentiation of angioblasts from hemangioblasts, which later form the inner lining of blood vessels. In the next step, Ang1 mediates the recruitment of pericytes surrounding the formed endothelial tubes and enables the vessels to form the primary capillary plexus (Hanahan, 1997; Risau, 1997; Lindahl et al., 1997). Angiogenesis is also initiated by VEGF. During this process, a previously formed capillary plexus is remodelled into distinguished arteries and veins. Later, this forms a mature vascular system, which is stabilized by TGFβ and PDGF that help in strengthening the extracellular matrix and recruitment of pericyte cells that contribute to the flexibility of the capillary wall (Hanahan, 1997; Risau, 1997; Lindahl et al., 1997) (Figure 1.1).

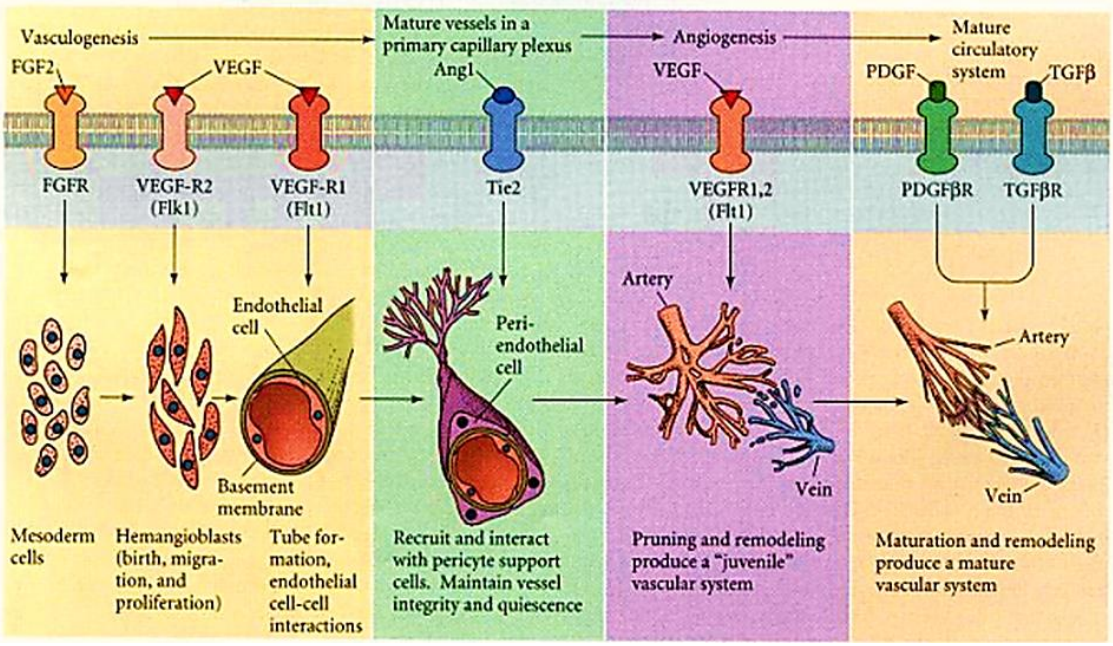


Figure 1.1 Vasculogenesis and angiogenesis. (Taken form Gilbert, 2000)

1.2 Transcription Factors involved in Vasculogenesis

Several transcription factor families play critical roles in vascular development and angiogenesis (Figure 1.2). Among the transcription factors that have been identified, are such factors as E26 transforming factor (Ets), Forkhead, Gata, and Kruppel-like families.

1.2.1 E26 Transforming Factor (ETS)

Ets proteins seem to play a central role. It has been shown that many endothelial genes are direct targets of Ets regulation (Dejana et al., 2007). Mouse transgenic studies have demonstrated important roles for Ets factors and essential Ets binding sites for vascular expression of Flk1, Flt1, Tie1, Tie2, Icam, von Willebrand factor, eNos, integrins, and VE-cadherin (Gory et al., 1998; Kappel et al., 2000; Korhonen et al., 1995; Lelievre et al., 2001; Meadows et al., 2009). Overexpression of the Ets factors Etv2, Erg or Fli1 in *Xenopus* embryos drives ectopic expression of endothelial marker genes (Baltzinger et al., 1999; Meadows et al., 2009; Meadows et al., 2011; Salanga et al., 2010; Neuhaus et al., 2010).

1.2.2 T-cell acute lymphocytic leukemia 1 (Tal1)

Tal1, also known as SCL (stem cell leukemia), is expressed during the development of hematopoietic and endothelial precursor cells. Tal1 has specific roles for hematopoietic differentiation, but it has also an important role in blood vessel development. Tal1 null mice die

at E9.5 due to an absence of erythropoiesis while endothelial marker expression was unaffected (Barton et al., 1998). Loss of Tal1 in *Xenopus* causes a severe reduction in the number of erythrocytes (Myers and Krieg, 2013). In zebrafish studies, misexpression of Tal1 induced ectopic expression of blood and endothelial markers suggesting Tal1 has a role in endothelial specification (Barton et al., 1998).

1.2.3 Forkhead (Fox)

In mammals, there are more than 100 identified members of the Forkhead (Fox) transcription factor family (Carter and Brunet, 2007). From this large family, at least four subfamilies, FoxC, FoxF, FoxH, and FoxO, have been identified to play important endothelial roles (De Val and Black, 2009). Fox has been identified as a cofactor for Ets proteins in the regulation of endothelial genes (De Val et al., 2008).

The knockout of FoxC1 and FoxC2 in mice results in embryonic lethality at E9.5 due to severe vascular defects (Seo et al., 2006). Loss of FoxF1 in mice is embryonic lethal during midgestation (Mahlapuu et al., 2001). In zebrafish loss of FoxH1 causes an upregulation of Flk1, while overexpression of FoxH1 decreases Flk1 expression (Choi et al., 2007). Also loss of Foxo1 in mice causes embryonic lethality by E10.5 due to defective vascular remodeling (Furuyama et al., 2004; Hosaka et al., 2004).

1.2.4 GATA

The six members of the GATA family of transcription factors can be divided into subfamilies based on sequence homology and expression patterns. GATA1/2/3 are expressed mainly in hematopoietic cells, while GATA4/5/6 are expressed in different mesoderm and endoderm-derived tissues (Song et al., 2009). GATA2-null mice die at E10 due to defects in the development of hematopoietic cells (Tsai et al., 1994). In *Xenopus* loss of GATA2 causes a loss of erythroid markers (Myers and Krieg, 2013). Loss of GATA3 in mice causes embryonic lethality between E11 to E12 due to an internal bleeding and defects in liver hematopoiesis (Pandolfi et al., 1995).

1.2.5 Krüppel-like factors (KLF)

KLFs, which are members of the zinc finger family of transcription factors, have critical roles in vascular biology. In mammals, from 17 known KLFs, 3 members, are expressed in endothelial cells, KLF2, KLF4, and KLF6 (Atkins and Jain, 2007). KLF2 null mice die between E12.5 to E14.5 due to hemorrhaging caused by weak vessel stabilization (Kuo et al., 1997). In *Xenopus* embryos KLF2 cooperates with the Ets family member Erg to activate Flk1 expression (Meadows et al., 2009).

1.2.6 LIM domain only 2 (Lmo2)

Lmo2 is a LIM domain protein that acts as a transcriptional cofactor. Rather than binding DNA, Lmo2 acts as a scaffold for transcription factors that enhance DNA binding or transcriptional activity. Studies in HUVEC cells and zebrafish embryos have demonstrated that Lmo2, Gata2, and Tal1 function together to activate VE-cadherin (Deleuze et al., 2007; Patterson et al., 2007). Lmo2 is an essential protein during blood development. Homozygous mutant mice fail to specify erythrocytes (RBC) in the yolk sac and die around E10.5 (Warren et al., 1994).

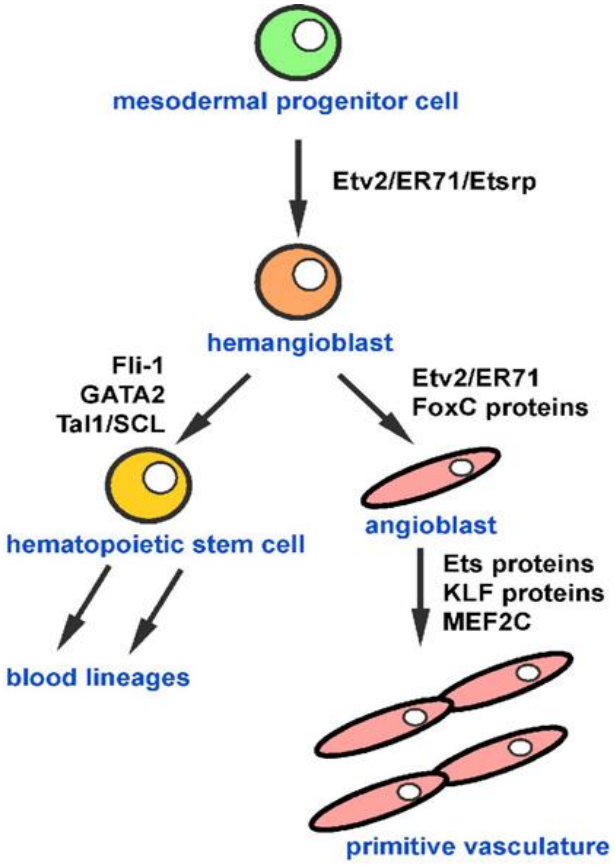


Figure 1.2 Different stages in endothelial development are regulated by different sets of transcription factors. The endothelial cells development from mesodermal progenitors and hemangioblasts to differentiated arterial, venous, and lymphatic endothelium and the various transcription factors associated with their development. Fli-1, GATA2, and Tal1 control differentiation of hematopoietic cells from hemangioblasts, while Etv2 and FoxC proteins control the differentiation of endothelial cells from that progenitor population and that Etv2 likely sits at the top of this transcriptional cascade. (Taken from De Val et al., 2009).

1.3 ETS Family Proteins

Approximately 30 Ets family genes have been identified in several vertebrate species with 27 and 26 Ets genes identified in the human and mouse genomes respectively (Bult et al., 2008; Hollenhorst et al., 2007). The majority of ETS factors activate gene transcription, but some ETS proteins can act as transcriptional repressors (Lelievre et al., 2001; Sharrocks, 2001). ETS proteins are characterized by a highly conserved DNA binding domain referred to as the ETS domain, which is a winged helix-turn-helix domain that binds to the core DNA sequence 5'-

GGA(A/T)-3'. The winged helix-turn-helix motif is about 85 amino acids long and consists of three alpha-helices and four anti-parallel beta-sheets. The third helix is responsible for DNA-binding specificity (Sharrocks et al., 1997). ETS proteins regulate various biological processes including cell proliferation, apoptosis and differentiation of multiple cell lineages (Oikawa and Yamada, 2003; Sharrocks, 2001). Most ETS factors are involved in different aspects of development and disease. Several studies have shown that ETS transcription factors are particularly important for development of the hematopoietic and vascular lineages. ETS proteins directly regulate expression of most, if not all, endothelial genes (Dejana et al., 2007).

A survey of the ETS family shows that at least 19 different ETS factors are expressed in human endothelial cells (Hollenhorst et al., 2004), while transcripts for 12 ETS factors are present in endothelial cells of zebrafish (Liu and Patient, 2008), and 7 Ets genes (Elk3, Erg, Ets1, Ets2, Etv2, Etv6 and Fli1) are highly expressed in embryonic endothelial cells (De Val and Black, 2009; Dejana et al., 2007; Lelievre et al., 2001; Salanga et al., 2010). Recent studies suggest that Etv2 is the most important of these genes during vasculogenesis (Meadows at el., 2011).

1.3.1 Etv2 (Ets variant 2), (Etrsp/ER71)

Recent studies in mouse, *Xenopus* and zebrafish demonstrate that ETV2 (Etrsp/ER71) is the most important transcriptional regulator of embryonic endothelial development. In mouse, Etv2 expression is detected, starting at E7.5, in the yolk sac blood islands region. Expression in the major blood vessels of embryos is observed from E8.25 to E9.5 (Lee et al., 2008). Etv2 expression in zebrafish is visible at 36 hpf in developing vessels (Liu and Patient, 2008; Sumanas and Lin, 2006). *Xenopus* ER71 is expressed after gastrulation in the forming blood islands. At later stages ER71 is strongly expressed in migrating angioblasts and in newly formed blood vessels throughout the embryo. At tadpole stages ER71 is no longer detectable (Salanga et al., 2010; Neuhaus et al., 2010).

ETV2 loss-of-function studies in *Xenopus* and zebrafish embryos show similar effects, with ETV2 morphant embryos showing a deficiency of angioblasts and vascular structures (Sumanas and Lin, 2006; Gomez et al., 2009; Salanga et al., 2010; Neuhaus, et al., 2010). ETV2 null mice have severe lack in blood and vascular structures (Lee et al., 2008).

Forced expression of ETV2 in *Xenopus* embryos results in ectopic activation of endothelial and hematopoietic markers (Neuhaus et al., 2010; Myers and Krieg, 2013). Similarly, the zebrafish ETV2 orthologue is sufficient to activate ectopic expression of endothelial genes (Sumanas and Lin, 2006; Gomez et al., 2009; Wong et al., 2009). However, the influence of ETV2 on hematopoiesis seems to vary between the different species. In mouse, ETV2 is essential for general hematopoiesis, while in zebrafish ETV2 is required for development of the myeloid but not the erythroid lineages (Lee et al., 2008; Sumanas et al., 2008). In *Xenopus* is appears that

ETV2 is not required for development of either the erythroid or myeloid lineage (Neuhaus et al., 2010; Salanga et al., 2010).

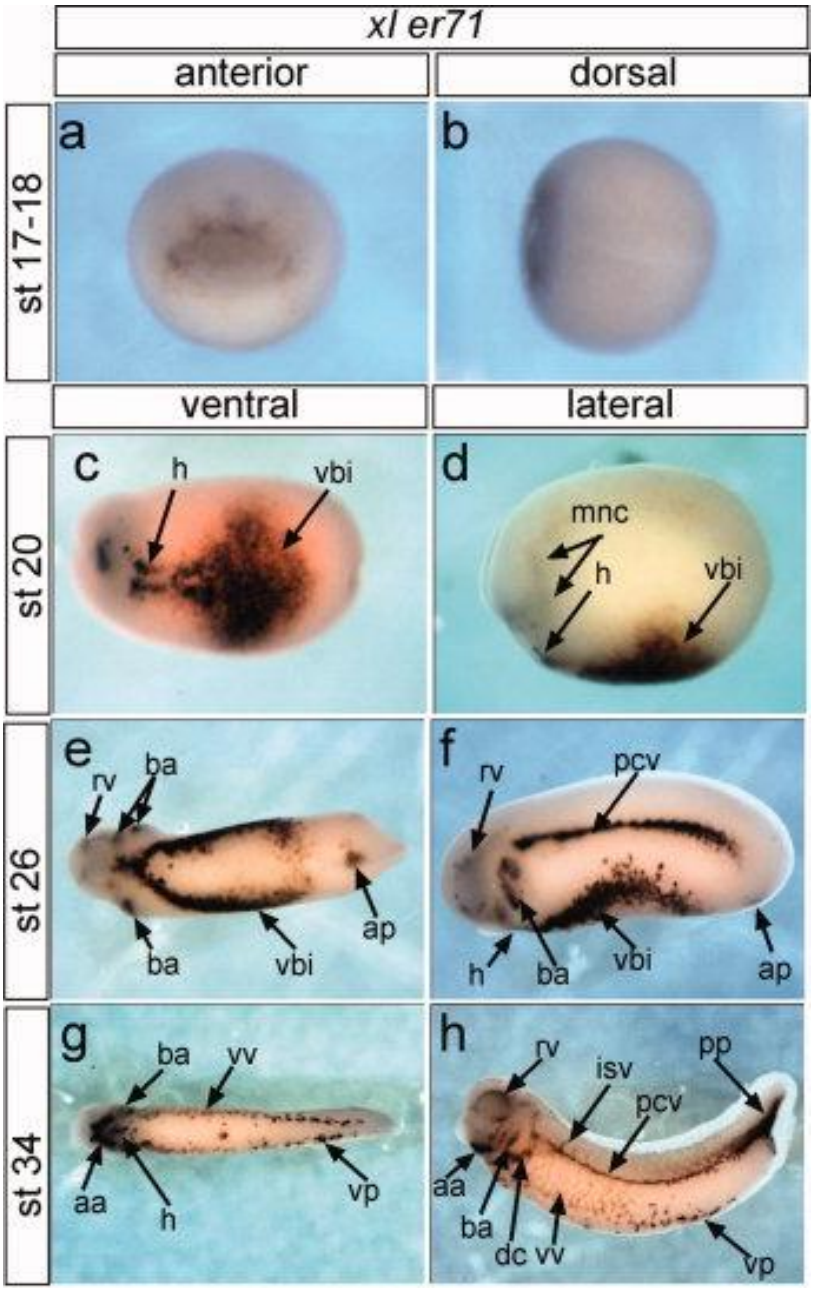


Figure 1.3 The expression of ER71 in *Xenopus* embryos. **A**: Whole-mount *in situ* hybridization of wild type embryos of different stages. ER71 is expressed in progenitor cells of the vascular system. **a, b**: ER71 expression at NF stage 17-18. ER71 is expressed in the anterior portion of the embryo in the vicinity of the cement gland. **c-h**: ER71 expression between NF stage 20 and 34. aa, anterior aorta; ap, anal porus; ba, branchial arches; dc, duct of cuvier; h, heart anlage; isv, intersomitic veins; mnc, migrating neural crest; pcv, posterior cardinal vein; pp, posterior precursors; rv, retinal vein; vp, ventral precursors; vbi, ventral blood islands; vv, vitelline veins. (Taken from Neuhaus et al., 2010).

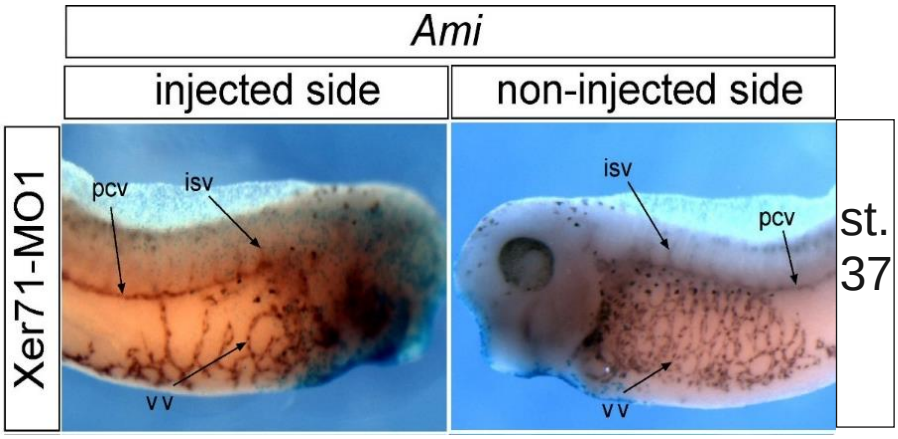


Figure 1.4 Knock-down of ER71 function leads to a severe reduction in the expression of Vascular marker gene *ami* (taken from Neuhaus, et al., 2010)

1.4 *Xenopus laevis* as a model organism to study the vascular system

The South African clawed frog, *Xenopus laevis* has long been a favorite organism for studying development because of its large egg size, extra-uterine development, and the ability of the embryos to heal easily after microsurgery. Many mysteries of vertebrate development have been analyzed by using this organism with typical experimental approaches, such as fate mapping, transplantation experiments and explant cultures. With *Xenopus* embryos, gain-of-function experiments can be quickly and easily performed by microinjection of *in vitro* synthesized RNA, DNA or proteins after fertilization. Inhibition of certain gene function can be achieved by injecting antisense morpholino oligonucleotides (MOs).

Animal cap explants resemble mammalian embryonic stem cells with respect to their pluripotency. They can give rise to derivatives of all three germ layers *in vitro*, when exposed to suitable signaling factors, and thereby provide a strong tool to study the molecular basis of embryonic induction and cellular specification. *Xenopus laevis* has specific advantages in studying the cardiovascular system (vasculogenesis and angiogenesis). It has more clear and differentiated cardiovascular system and embryos allow visualization of almost the entire vascular network. It has been demonstrated that it possesses a higher degree of similarity with higher vertebrates than other model organisms such as zebrafish. *Xenopus laevis* embryos can survive till tadpole stage without a functional circulatory system whereas mammalian and chick embryos cannot (Copenhaver, 1926). It shows similarities with human embryos (Evans et al., 1912) in having an artery and a vein at each intersomitic vessel whereas zebrafish embryos shows random pattern (Isogai et al., 2001). Posterior cardinal veins first develop as paired vessels, which then fuse starting at the tail and extending towards anterior region (Levine et al.,

2003) whereas zebrafish shows a single medial posterior cardinal vein (Isogai et al., 2001). It shows separated atria, lungs, defined heart valves, a mammalian like pattern of tail vasculature, blood islands and a vitelline network (Kolker et al., 2000; Mohun et al., 2000; Cleaver et al., 1999). Recent studies discovered several genes like (*ami*, *lmo2*, *cdh5* and *mpo*) which are specific cardiovascular and hematopoietic markers (Inui and Asashima, 2006; Neuhaus et al., 2010). They were used in different experiments to explore the expression of these markers in *Xenopus* embryos during vascular system development.

1.5 Aim of the thesis

Several studies have shown that ER71 is the most important member of the ETS family involved in blood vessel formation. Loss of ER71 function leads to a strong reduction of vascular structures during embryonic development in *Xenopus*, mice and zebrafish. As was reported in Neuhaus et al., 2010, overexpression of ER71-mRNA during early *Xenopus* development causes gastrulation problems making the analysis of ER71 overexpression during vessel formation impossible. Using the animal cap system Neuhaus et al. could show that ER71 is able to induce several known vascular and hematopoietic genes. In the project of this thesis, we wanted to do overexpression experiments with ER71 in order to identify potential target genes of ER71. Furthermore we wanted to use inducible ER71 expression constructs to avoid gastrulation problems, which would allow to investigate the effect of ER71 overexpression on early and later stages of blood vessel development.

2. Materials

2.1 The experimental animal - *Xenopus laevis*

The South African clawed frog *Xenopus laevis* is an amphibian of the order Anura and has a natural geographic range along the African Rift Valley, south of the Sahara Desert. Pigmented and albino frogs were obtained from a commercial supplier (NASCO, USA) and held in aquaria (water temperature 19°C).

2.2 Bacteria

E. coli XL1-Blue (Stratagene GmbH, Heidelberg)

2.3 Chemicals

Acetic acid	Roth
Acetic anhydride	Sigma
Agarose	Roth
Albumin Fraction V	Roth
Ammonium Persulfate	Serva
Ampicillin sodium salt	AppliChem
Bovine Serum Albumin (BSA)	Sigma
Blocking reagent	Roche
5-Bromo-4-chloro-3-indolyl-phosphate (BCIP)	Fermentas
Bromphenol blue sodium salt	Merck
β-Mercaptoethanol	Sigma
Calcium chloride, dihydrate	AppliChem
Calcium sulfate	Roth
CHAPS	Roth
Chloroform	Merck
DIG RNA labeling mix	Roche
DNA Ladder, O'GeneRuler ™ 1kb	Fermentas
DAPI	Roth
Dexamethasone	Sigma
Ethidium Bromide	Q-Biogene
Ethylenediaminetetraacetic acid (EDTA)	Sigma

Ethlene glcycol-bis (2-amino-ethylether -N,N,N',N')-tetra-acetic acid (EGTA)	Sigma
L-Cysteinhydrochloride	Roth
10 mM dNTP mix	Fermentas
Fast Red	Roche
Formamid	Roth
Formaldehyde	Roth
Glycerol	Roth
Horse Serum (HS)	Gibco
Human chorionic gonadotropin (HCG)	Sigma
Isopropanol	Roth
LB Broth Base	Invitrogene
LB Agar	Invitrogene
Lipofectamine 2000	Invitrogen
Lithium chloride	Roth
L-[^{35} S]-Methionin	Bioscience
Ethanol	Roth
MOPS	Biogene
Mowiol	Calbiochem
Nitro blue tetrazolium chloride (NBT)	Fermentas
Nile blue chloride	Fluka
NTP set (100 mM for each separately)	Fermentas
Paraformaldehyde	Roth
Potassium hexacyano-ferrate (III) (K_3 Fe $(CN)_6$)	Sigma
Potassium hexacyano-ferrate (II), trihydrate (K_4 Fe$(CN)_6$ $3H_2O$)	Sigma
Potassium chloride	Roth
Potassium hydrogenphosphate	Roth
ProteinaseK	Merck
Red-Gal (5-Bromo-6-chloro-3-indolyl-β-D-galactopyranoside)	Sigma
RNase OUT^{TM} Ribonulease Inhibitor	Invitrogen
RNase A	Fermentas
RNase T1	Sigma
Sodium acetate	Roth
Sodium azide	Roth
Sodium bicarbonate	Sigma
Sodium chloride	Roth
Sodium citrate	Fluka

Sodium dihydrogenphosphate	Merck
Sodium dodecyl sulfate (SDS)	Roth
Sodium hydrogenphosphate, dodecahydrate	Merck
Sodium hydroxide	Roth
Sucrose	Roth
Torula RNA	Sigma
Triethanolamine	Roth
Tris(hydroxymethyl)-aminomethane (Tris)	Roth
Triton X-100	Ferak
TRIzol® Reagent	Invitrogene
Tween-20	Roth
X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)	Q-Biogene
Xylencyanol	Roth

2.4 Buffers, solutions and media

2.4.1 Embryos preparation

Human chorionic gonadotropin (HCG)

10,000 U/vial HCG (Sigma) was suspended in 5 ml ddH_2O to make a stock solution of 2000 U/ml. The solution was aliquoted and stored at -20 °C.

5x MBS (Modified Barth's Saline)

440 mM NaCl, 12 mM $NaHCO_3$, 5 mM KCl, 4.1 mM $MgSO_4$, 50 mM Hepes in dH_2O, pH adjusted to 7.4 then supplied with 2.05 mM $CaCl_2$. The solution was autoclaved and stored at room temperature. Upon requirement, the stock solution was diluted to 1x MBS or 0.1x MBS with distilled water.

L-Cystein hydrochloride solution (2 %)

2% L-Cystein hydrochloride in dH_2O, pH adjusted to 7.8–8.0.

Nile blue solution

Phosphate buffer containing 50 mM Na_2HPO_4 and 50 mM NaH_2PO_4 was warmed up to 60°C. 0.01% (w/v) Nile blue chloride was dissolved in it with stirring overnight. After filtration, the Nile blue solution was ready to use.

10x MEM (MOPS/EGTA/Magnesium sulfate buffer)

1 M MOPS, 20 mM EGTA, 10 mM $MgSO_4$ in dH_2O. The solution was filtrated with 0.2 µm filters and stored at room temperature.

MEMFA (MOPS/EGTA/Magnesium sulfate/formaldehyde buffer)

3.7% formaldehyde in1x MEM, prepared before use.

10x PBS (phosphate-buffered saline)

1.37 M NaCl, 27 mM KCl, 80 mM Na_2HPO_4 and 18 mM KH_2PO_4 in dH_2O, pH was adjusted to 7.4.with NaOH and autoclaved.

X-Gal stock solution

40 mg/ml X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) in DMSO, stored in the dark at -20°C.

X-Gal staining solution

5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 5 mM $MgCl_2$, and 1 mg/ml X-Gal in PBS.

500x Dexamethasone solution

20 mM dexamethasone in ethanol, stored at -20°C in the dark.

2.4.2 Whole-mount *in situ* hybridization

DEPC (Diethylpyrocarbonat) H_2O

0.1% (v/v) DEPC in ddH_2O was incubated at 37°C for 2 hrs and autoclaved.

PTw

0.1% Tween-20 in PBS.

Triethanolamine solution

0.1 M Triethanolamine-hydrochloride in dH_2O, pH adjusted to7.5.

100x Denhardt´s solution

2 % BSA, 2 % PVP and 2 % Ficoll 400 in dH_2O, stored at -20°C.

Torula RNA (10 mg/ml)

10 mg/ml Torula RNA in DEPC H_2O was dissolved at 37°C with shaking overnight. After centrifugation at 6000 rpm for 10 min, the supernatant was aliquoted and stored at -20°C.

20x SSC (standard saline citrate buffer)

3 M NaCl and 0.3 M Na Citrate in dH_2O, pH adjusted to 7.2-7.4, using citric acid.

Hybridization mix

50% formamid, 1 mg/ml Torula-RNA, 10 µg/ml Heparin, 1x Denhardt's, 0.1% Tween-20, 0.1% CHAPS, and 10 mM EDTA in 5x SSC, stored at -20°C.

NBT solution

100 mg/ml NBT in 70% DMF, stored at -20°C.

BCIP solution

50 mg/ml in 100% DMF, stored at -20°C.

Ethanol series

100%, 75%, 50% and 25% (v/v) ethanol in dH_2O respectively.

5x MAB (maleic acid buffer)

500 mM maleic acid, 750 mM NaCl in dH_2O, pH 7.5, autoclaved.

Boehringer Blocking Reagent (BMB) stock solution

10 % BMB was dissolved 1x MAB at 60°C, autoclaved and stored at -20°C.

MAB/BMB

2% BMB in 1x MAB.

MAB/BMB/HS

2% BMB, 20% heat-treated horse serum in 1x MAB.

APB (Alkaline phosphatase buffer)

100 mM Tris-HCl, pH 9.0, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween-20 in dH_2O.

Color reaction solution

1.75 µg/ml NBT and 3.5 µl/ml BCIP in APB.

TE buffer (Tris/EDTA buffer)

10 mM Tris-HCl (pH 7.5) with 1 mM EDTA.

RNase A stock solution

10 mg/ml of RNase A dissolved in TE buffer, heated at 100°C for 10 min, and stored at- 20°C.

Bleaching solution

50% (v/v) formamid and 1% H_2O_2 were dissolved in 5x SSC.

Solution A

50 mM Glucose and 25 mM Tris, pH 8.0 with 10 mM EDTA dissolved in ddH_2O.

Solution B

0.2 M NaOH and 1% SDS mixed with ddH_2O.

Solution C

30 ml 5M KAc pH 5.2 and 5.75 ml glacial acetic acid mixed with ddH_2O.

2.4.3 Vibratome sectioning

Gelatine-Albumin

0.44% (w/v) Gelatine, 13.5% (w/v) Albumin (Sigma) and 18% (w/v) Sucrose in PBS, stirred at 60°C till well dissolved and centrifuged at 6000 rpm for 10 min. Stored at -20°C.

2.4.4 Gel electrophoresis

10x TBE buffer (Tris/boric acid/EDTA buffer)

0.89 M Tris, 0.89 M boric acid and 20 mM EDTA were dissolved in dH_2O.

Glycerol loading buffer

10 mM EDTA, 30% glycerol (v/v), 0.025 % Bromphenol blue and 0.025 % Xylencyanol in 10 mM Tris-HCl, pH 7.5.

2.4.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Ammonium persulfate stock solution

10% (w/v) ammonium persulfate in dH_2O and stored at -20°C.

Tris-glycine electrophoresis buffer

25 mM Tris base, 250 mM glycine and 0.1% SDS in dH_2O, pH 8.3.

2x SDS gel loading buffer

200 mM DTT, 4% (w/v) SDS, 0.2% bromophenol blue and 20% (v/v) glycerol in 100 mM Tris-HCl, pH 6.8. Aliquots were stored at -20°C.

2.4.6 Immunostaining (Embryos/Cells)

4% Paraformaldehyde (PFA)

4% Paraformaldehyde in PBS, stirred and heated to 60-65°C till the solution became clear, pH adjusted to 7.2. Aliquots were stored at -20°C.

Permeabilization and blocking solution

20 mg /ml bovine serum albumin (BSA, Roth) and 0.5% (v/v) Triton X-100 in PBS.

Antibody buffer

10 mg/ml BSA and 0.05% Triton X-100 in PBS.

PBS-TB

0.05% (v/v) Tween-20 and 0.2% BMB in PBS.

PBS-TBN

0.05% (v/v) Tween-20, 0.2% BMB and 0.3 M NaCl in PBS.

2.4.7 Media

Luria-Bertani (LB) medium

20 g LB Broth Base was dissolved into 1 l dH_2O and autoclaved for 20 min at 121ºC, stored at 4ºC.

Luria-Bertani (LB)-Ampicillin (Amp) agar plate:

32 g LB Agar was dissolved in 1 l dH_2O and autoclaved for 20 min at 121°C. After the medium was cooled down to around 50°C, ampicillin solution (100 mg/ml in dH_2O) was added with a final concentration of 100 µg/ml and plates were poured in a sterile hood.

Cell culture medium (DMEM)

10% Fetal calf serum (FCS), 1%Antibiotics/L-glutamine.

Trypsin solution

1xPBS, 0.05 % Trypsin and 0.2 % EDTA.

2.5 Antibodies

Anti-Digoxigenin/AP (Roche Diagnostics)

Fab fragment of polyclonal antibodies from sheep specifically recognizing digoxigenin and digoxin, conjugated with alkaline phosphatase.

Anti-rabbit/AP (Sigma-Aldrich)
An alkaline phosphatase (AP)-conjugated goat affinity purified antibody to rabbit IgG (whole molecule).

Anti-Myc-Tag/AP (Cell Signaling)
Monoclonal antibody generated from mouse specifically recognizing the Myc-tag of targeted proteins in transfected cells.

Anti-Fluorescein/AP (Roche)
Fab fragment of polyclonal antibodies from sheep recognizing fluorescein labeled nucleic acids in cells and tissues, conjugated with alkaline phosphatase.

2.6 Enzymes

Restriction enzymes with supplied buffers	Fermentas
Terminal Deoxynucleotidyl Transferase (TdT, 20 U/µl) with supplied buffer	Fermentas
RNase A	Sigma-Aldrich
RNase T1	Sigma-Aldrich
Proteinase K	Merck
T4 DNA-Ligase (3 U/µl) with supplied buffer	Fermentas
SP6 RNA-Polymerase (50 U/µl) with supplied buffer	Stratagene
T3 RNA-Polymerase (50 U/µl) with supplied buffer	Stratagene
T7 RNA-Polymerase (50 U/µl) with supplied buffer	Stratagene
Taq DNA-Polymerase (5 U/µl) with supplied buffer	Fermentas
Pfu DNA-Polymerase (2.5 U/µl) with supplied buffer	Fermentas
Deoxyribonuclease I (DNaseI, RNase-free) (1 U/µl)	Fermentas

2.7 Kits

The following kits were used in this study, according to the manufacturers' instructions:

Big Dye Terminator Cycle SequencingKit	Applied Biosystems
mMESSAGE mMACHINE® SP6	Ambion
pGEM®-T Vector System	Promega
QIAGEN® PCR Purification Kit	Qiagen
QIAGEN® Plasmid Midi Kit	Qiagen

QIAEX® Gel Extraction Kit	Qiagen
RNeasy Mini Kit	Qiagen
Super Script™ II Reverse Transcriptase Kit	Invitrogen
TnT®-Coupled Reticulocyte Lysate System	Promega

2.8 Oligonucleotides for PCR

The oligonucleotides were ordered from Sigma-Aldrich and dissolved in ddH_2O to get a 100 µM stock solution. In the following sequences, f represents forward primer, and r represents reverse primer, "seq" indicates the primer is used for sequencing

Xl-*er71*-f1-EcoRI	5'-CGAATTCAATGGATCCCAGTATC-3'
Xl-*er71*-r1107-XhoI	5'-CGCTCGAGTTGAATCCTGGATCTCTGGGTTTTGG-3'
	28 Cycles
hGR-EcoRI-mut	5'-GGATAAGACCATGAGTATTGAGTTCCCCGAGATGTTAGCTG-3'
Xl-*er71*C320T-mut	5'-GCCATGTGATGAATTTCTGCCCTCTTTCCAGACG-3'
Xl-*er71*-ATG	5'-CCTCTCGAGATGGATCCCAGTATCTACTACTGC-3'
Xl-*er71*-UTR-f	5'-CAGAGATTTGGTGCAAGCAG-3'
Xl-*er71*-Exon 1-r	5'-ACTGCAGCTCTCAGGCTCTC-3' 30 Cycles
Xl-msr-246-f	5'-TCTCAGGGAATGG AGTGGTC-3'
Xl-msr-494-r	5'-GATCAAAGCTGAG GCAGGTC-3' 28 Cycles;
Xl-*er71*-f-1654	5'-TCCGGAGCAGAT CCATTAAC-3'
Xl-*er71*-r-1807	5'-CTTCATCGGAATG TGTGTGG-3' 30 Cycles
Xl-AMI-558-f	5'-ATGTCTGTAATCG CCGGAAC-3'
Xl-AMI-708-r	5'-CGATATCCGCCCT GTACAAT-3' 28 Cycles;
Xl-ami-rt-f202	5'-TAAATGGGTGCT GAGTGCAG-3'
Xl-ami-rt-r577	5'- GTTCCGGCGATT ACAGACAT-3' 28 Cycles
Xl-fli1-rt-f848	5'-TGAATAAAAGCGG GGATCAG-3'
Xl-fli1-rt-r1051	5'-CTTCATCTGGGT CGGTCATT-3' 28 Cycles
Xl-vegf-b-rt-f433	5'-GCCATCTACGA ACCAGGTGT-3'
Xl-vegf-b-rt-r770	5'-AAATGTGGGAT TGGGAATGA-3' 28 Cycles
Xl-lmo2-rt-f358	5'-ATCGGGGATCGC TATTTCTT-3'
Xl-lmo2-rt-r508	5'-CCGAACAGCCT GAGGTAGTC-3' 28 Cycles
Xl-mpo-rt-f1197	5'-CCTACCTGCCCT TTGGTACA-3'
Xl-mpo-rt-r1359	5'-TCTCTGGCTAT GCGATTGTG-3' 28 Cycles
Xl-msr-rt-f603	5'-CCAAGTCTGATT CTGCGTGA-3'
Xl-msr-rt-r899	5'-GAAAGGCAGCCA GCAGATAG-3' 28 Cycles

Xl-runx1-rt-f933	5’-CCGCAGCTTTT AATCCTCAG-3’
Xl-runx1-rt-r1094	5’-TTGCCCTTCC TGGAGATATG-3’ 28 Cycles
Xl-SCL-rt-f799	5’-CTTTGGTGACCC AGACACCT-3’
Xl-SCL-rt-r1168	5’-CGGAGACAGCA TGTCTTGAA-3’ 28 Cycles
Xl-SPIB-a-rt-f526	5’-CAGGGGAATG CATTGGTTAC-3’
Xl-SPIB-a-rt-r746	5’-GGCCATTTTCT GGTAGGTCA-3’ 28 Cycles
XT-ve-cadherin-Ex10	5’-ATTCTGTG AGGAGGCTGGAA-3’
XT-ve-cadherin-Ex11	5’-CGCCTTCCT CATCATAGGTG-3’ 28 Cycles
Xl-exo3-RT-F-E	5’-GAGCAGCAATGTGTGAATGC-3’
Xl-exo3-RT-R-E	5’-CAGAGAAGCATCGTAAACAGG-3’ 28 Cycles
Xl-Egfl7-RT-F742	5’-GTGCATCAACTCTGCTGGAA-3’
Xl-Egfl7-RT-R967	5’-AAACGGAGTCAGCAGCAAGT-3’28 Cycles
Xl-camk2b-RT-F494	5’-TTGCATCCAGCAGATTCTTG-3’
Xl-camk2b-RT-R670	5’-AATCCAAACCAAGCCTGTTG-3’28 Cycles
Xl-grip-RT-F323	5’-CTGTGAATGGCATCAACCTG-3’
Xl-grip-RT-R536	5’-TCCTCATGTGCTCCTCCTCT-3’28 Cycles
Xl-sipa-RT-F17	5’-GGTGACAGTGAGGAGGATTC-3’
Xl-sipa-RT-R188	5’-CATTTAGCACAATGAAGTCC-3’28 Cycles
Xl-cplx2-RT-F101	5’-GATGCGAATTTCCCTGTGAC-3’
Xl-cplx2-RT-R304	5’-GCATACTTAGCCTTCCGTTC-3’28 Cycles
Xl-ODC-1-f	5’-GCCATTGTGAAGACTCTCTCCATTC-3’
Xl-ODC-1-r	5’-TTCGGGTGATTCCTTGCCAC-3’ 26 Cycles
SP6-seq	5’-TTTAGGTGACACTATAGAATAC-3’
T7-seq	5’-TAATACGACTCACTATAGGGCGA-3’
T3-seq	5’-ATTAACCCTCACTAAAGGGA-3’

2.9 Vectors and Constructs

2.9.1 Vectors

pGEM®-T (Promega)

pGM-T vector contains a 3' terminal thymidine overhang at both ends and is convenient for the cloning of PCR products. The PCR fragments with a 3'-termianl deoxythymidine could be directly cloned into pGEM®-T vector. It contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of β-galactosidase (Figure 2.1).

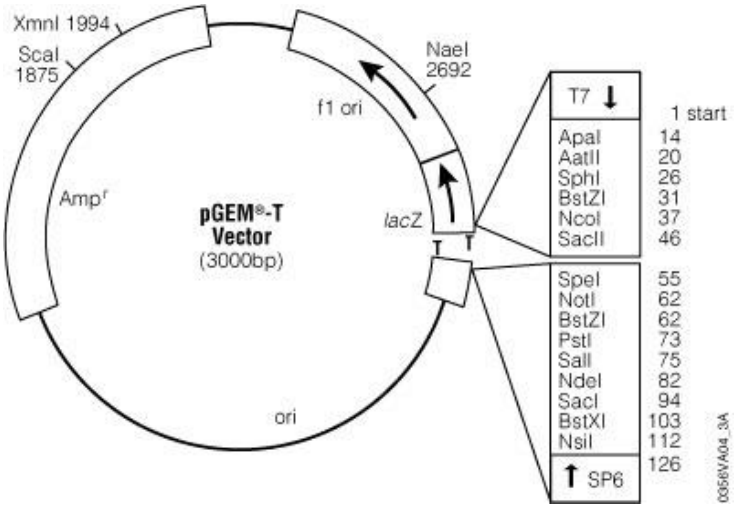


Figure 2.1 pGEM®-T Vector circle map

pCS2+

This multipurpose expression vector contains a strong enhancer/promoter (simian CMV IE94) followed by a polylinker (polylinker I) and the SV40 late polyadenlyation site. The SP6 promoter allows *in vitro* RNA synthesis of sequence cloned into polylinker I. The second polylinker (polylinker II) provides several possible sites to linearize the vector for SP6 RNA transcription. This vector was used for the generation of constructs for *in vitro* synthesis of sense mRNA. The graphic map is shown in Figure 2.2. The interested genes were cloned into polylinker I to make the constructs. After linearized with a restriction enzyme in polylinker II, the constructs could be used as the template for *in vitro* synthesis of the sense RNA (Turner and Weintraub, 1994).

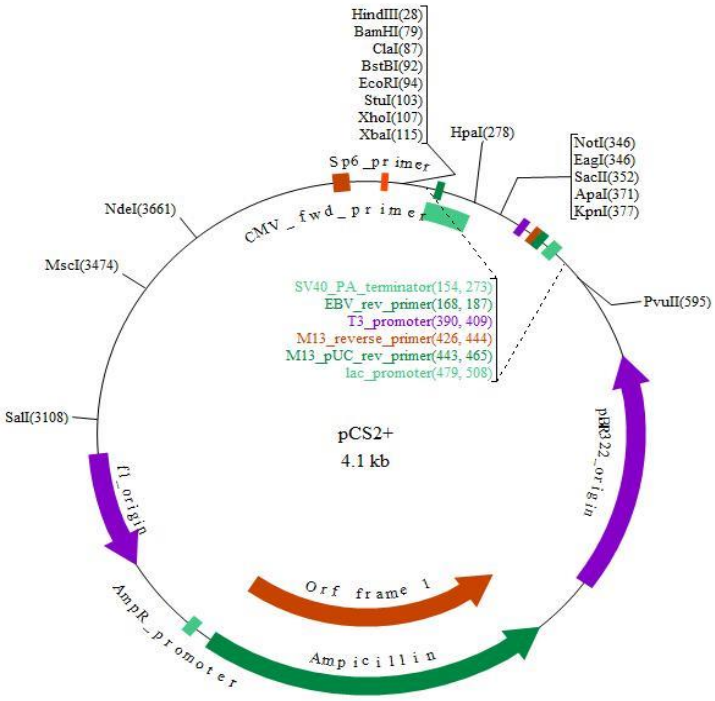


Figure 2.2 pCS2+ vector graphic map

2.9.2 Constructs

The antisense probes generated from the following constructs are all specific for *Xenopus* transcripts.

Ami-SK/pBlueScript SK II

This construct was linearized with *Eco*RI and *in vitro* transcribed with T7 to synthesize Ami antisense probe for whole-mount *in situ* hybridization.

Lmo2/pCMV-Sport 6

This construct was linearized with *Eco*RI and *in vitro* transcribed with T7 to synthesize Lmo2 antisense probe for whole-mount *in situ* hybridization.

2.10 Equipments

Microliter pipettes

Transferpette® S, Model D-10	BRAND GMBH+CO KG, Germany
Transferpette® S, Model D-100	BRAND GMBH+CO KG, Germany
Transferpette® S, Model D-1000	BRAND GMBH+CO KG, Germany

PCR Thermocycler

Tpersonal Thermocycler	Biometra, Germany
TGRADIENT Thermocycler	Biometra, Germany

Centrifuge

Biofuge pico	Heraeus, Germany
SIGMA 2K15	Sigma Laborzentrifugen, Germany
Sorvall RC-5B	Thermo Scientific, USA

Spectrophotometer

NanoDrop® Spectrophotometer ND-100	peqlab Biotechnology, Germany
Bio photometer	Eppendorf, Germany

Elektroporator

Electro Square Porator™ ECM830	BTX, Germany

Sterile Hood

KS12	Thermo Scientific, USA

Incubator/Thermoblock/Waterbath

Incubator: Function line	Heraeus Instruments,Germany
Incubator shaker: innova™4300	New Brunswick Scientific, USA
Incubator shaker: innova™4230	New Brunswick Scientific, USA
Water bath DIN 40050-IP20	Memmert, Germany
Thermomixer: Thermomixer 5437	Eppendorf, Germany
Thermomixer: HTMR-131	HLC-HaepLaborConsult,Germany

Shaker

Rocky 100	Labortechnik Fröbel, EU
RM5V-30	CAT. M. Zipperer, Germany

Histological equipments

Vibratome Leica VT1000 S	Leica Microsystem, Germany
Cover slides (24x 60 mm)	Menzel-Gläser, Germany

Electrophoresis

Electrophoresis power supply E844	Consort, Belgium

Power Pack P25	Biometra, Germany
Bio-Rad Gel Doc 2000	Bio-Rad Laboratories, USA
Microinjection	
Microinjector: PV820 Pneumatic Picopump	Helmut Saur, Germany
Needle-puller: PN-30	Narishige, Japan
Microscope	
Zeiss Stemi 2000	Carl Zeiss, Germany
Olympus SZX12	Olympus Microscopy, Japan
Leica DMR	Leica Microsystem, Germany
Nikon Eclipse E600	Nikon, Japan
Camera	
iNTAS MS 500	iNTAS, Germany
Vosskühler CCD-1300QLN	Vosskühler, Germany
Computer	
Personal Computer	hp, China
Software	
BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)	National Institute for Health, USA (Altschu et al., 1997)
Microsoft® Office 2013	Microsoft, USA
Photoshop elements 10.0	Adobe Systems, USA
Primer3 (http://frodo.wi.mit.edu/)	Whitehead Institute for Biomedical Research USA (Steve Rozen and Helen J.Skaletsky, 2000)
QCapture Pro 5.1	QImaging, USA

3. Methods

3.1 Genetic methods

3.1.1 Construction of *Xenopus* ER71

The nucleotide sequences of ER71 cDNA were obtained from the NCBI-Genbank, the accession number (NM_001096131) encodes a putative protein of 369 amino acid residues which contains a highly conserved ets-domain at the C-terminus.

3.1.2 Cloning

For cloning ER71, PCR was carried out in a 50 µl reaction mix containing 5 µl 10x buffer (supplied with enzyme), 10-30 ng DNA template, 0.25 µM each of forward and reverse primers, 0.5 mM dNTPs and 1 µl Taq DNA polymerase. The thermocycle program was performed with activating the enzyme and denaturing the DNA template at 95°C for 2 min, followed by 26 cycles of DNA denaturation at 95°C for 30 sec, annealing at 55-60°C for 30 sec and extension at 68°C for 1-3 min according to the length of the PCR product and the final extension at 72°C for 10 min.

ER71/pGMT

The ER71-cDNA, was amplified by PCR using Xl- *e*r71-f1-EcoRI and Xl- *e*r71-r1107-*Xho*I primers and the obtained fragment was subcloned into the pGMT vector.

ER71-GR/pCS2+

Rxl-GR/pCS2+ plasmid was digested with *Eco*RI/ *Xho*I to take the Rxl fragment out and fuse ER71-cDNA into GR/pCS2+. This construct was linearized with *Not*I and transcribed with SP6 to synthesize the sense RNA for overexpression in embryos and animal caps.

ER71-Myc-GR/pCS2+

Myc-GR/pCS2+ vector was digested with *Eco*RI/ *Xho*I and ER71-cDNA fragment subcloned into the vector. This construct was used for cell transfection and immunostaining. To detect the fusion protein (ER71-Myc-GR) in HeLa cells and animal caps.

3.1.3 Preparation of electrocompetent bacteria

A single colony of *E. coli* XL-1 Blue was picked from a LB plate containing ampicillin inoculated in 3 ml LB medium without antibiotics, and cultured overnight at 37°C with a rotary speed of 220 rpm. This 3 ml bacteria culture was then inoculated to 300 ml LB medium without

antibiotics in a 1 l flask, and cultured at 37°C with a rotary speed of 220 rpm for about 3 hrs until the OD reached approximate 0.5. The culture was intensively cooled down on ice. Meanwhile, the centrifuge cups, pipets and 1.5 ml eppendorf tubes supposed to be used in the preparation were all pre-cooled at 4°C.

The bacteria were transferred to a pre-cooled centrifuge cup and precipitated by centrifugation at 6,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was gently resuspended in chilled 10% glycerol (autoclaved) and collected again by centrifugation at 6,000 rpm for 20 min. The washing step with chilled 10% glycerol was repeated three more times and the pellet was finally resuspended in 2 ml 10% glycerol. The bacteria were aliquoted in 50 µl per 1.5 ml Eppendorf tube on ice and immediately transferred to liquid Nitrogen. Aliquots were stored at -80°C.

3.1.4 Electroporation

1 µl circular plasmid or 2 µl ligated plasmid was added to 50 µl electrocompetent bacteria and gently mixed by tapping. After incubation on ice for 5 min, the cell-DNA mixture was transferred to a chilled 1 mm electroporation cuvette (Equibio, UK) and applied on the electroporator (Electro Square Porator^{TM} ECM830, BTX). The sample was pulsed once (500 V for 8 msec) and immediately filled with 450 µl chilled LB medium. After being gently mixed by pipetting, the bacteria were kept on ice. These 500 µl bacteria were transferred to a 1.5 ml eppendorf tube and incubated at 37°C for 30 min. A 50 µl aliquot was kept and the rest was spread on LB-Amp plates respectively, and incubated overnight at 37°C.

3.1.5 Colony PCR

A single colony was picked with an autoclaved toothpick from a LB-Amp plate and scratched on a fresh LB-Amp plate. The rest bacteria on the toothpick were rinsed in 10 µl ddH_2O. This 10 µl bacteria suspension was heated at 95°C for 10 min to lyse the bacteria, and 8 µl of it was used as the template for the colony PCR. A standard 25 µl colony PCR reaction contained 8 µl of the template, 1.5 mM $MgCl_2$, 2.5 µl Taq polymerase buffer (supplied with enzyme, without $MgCl_2$), 1 µM forward primer and reverse primer respectively, 0.1 mM dNTPs and 0.1 µl Taq polymerase (5 u/µl, Fermentas).

The PCR reaction was run under a thermocycle program with activating the enzyme and denaturing the DNA template at 95°C for 2 min, followed by 26 to 30 cycles of DNA denaturation at 95°C for 30 sec, annealing at 55-60°C for 30 sec and extension at 68°C for 30 sec to 2 min according to the length of the PCR product (1kb/1min as recommended by the manufacturer), and the final extension at 72°C for 10 min. The PCR products were analyzed on a 1% agarose gel marked with 1 kb DNA Ladder (Fermentas).

3.1.6 Plasmid preparation

3.1.6.1 Plasmid mini-preparation

The bacteria were grown in 3 ml LB medium containing appropriate antibiotics overnight at 37°C. 1.5 ml of the bacteria culture was collected in an eppendorf tube and centrifuged at 14,000 rpm for 30 sec in a bench centrifuge. The supernatant was removed and the pellet was resuspended in 100 µl of solution A, and incubated on ice for 5 min, than 200 µl of solution B was mixed with the suspension, incubated on ice for 5 min. 150 µl of solution C was added in the bacteria suspension, and mixed thoroughly. After incubation on ice for 5 min, the bacteria lysate was centrifuged for 10 min at 14,000 rpm and 400 µl of the suspension was taken into fresh 1.5 ml centrifuge tube, 1 ml of 96% ethanol was added and incubated at room temperature for 5 min. After centrifugation at room temperature for 3 min, the supernatant was discarded and the pellet was washed with 200 µl of 70% ethanol by centrifuging at full-speed for 5 min at room temperature. The supernatant was removed. After the pellet was air-dried, it was dissolved in 20 µl of TE buffer with RNase A (10 µg RNase A per ml TE).

3.1.6.2 Plasmid midi-preparation

When 1 µg/µl or a higher concentration of plasmid was required, the plasmid was extracted with a QIAGEN® Plasmid Midi Kit according to the manufacturer's manual.

3.1.7 Preparation of sequencing samples

For preparation of the template for sequencing reaction, the plasmid prepared as mentioned above, 50-100 ng/µl of the plasmid was required in a total volume of 15 µl ddH_2O, than submitted for the nucleotides sequencing assay to eurofins mwg/operon company (Ebersberg).

3.1.8 *In vitro* synthesis of sense RNAs

To prepare synthetic capped RNA, the SP6 mMessage-mMachine™ Kit (Ambion) was used according to the manufacturer's protocol. A 20 µl reaction contains 1-1.5 µg linearized plasmid template, 2 µl 10x reaction buffer, 10 µl 2x NTPs/Cap, 2 µl enzyme mix. Transcription was carried out at 37°C for 2.5 hrs. The DNA template was removed by addition of 2 U DNaseI followed by incubation at 37°C for 30 min. The mRNA was purified with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and eluted with 20 µl RNase-free H_2O. The concentration of synthesized RNA was determined using the NanoDrop® Spectrophotometer ND-1000 (peq lab, Germany), and the quality was examined on a 1% agarose gel. The synthesized RNA was stored in aliquots at -20°C.

3.1.9 *In vitro* synthesis of anti-sense RNAs

The preparation of digoxigenin-labeled antisense RNA was carried out in a 20 µl reaction mixture containing 1-1.5 µg linearized template plasmid, 2 µl 10x Transcription buffer (Fermentas), 1 µl RNase OUT™ (Invitrogen), 2 µl RNA polymerase (Fermentas), and 2µl Digoxigenin-Mix (a mix of 10 mM ATP, 10 mM GTP, 10 mM CTP, 6.5 mM UTP, and 3.5 mM Dig-11-UTP, Roche) in 12 µl ddH_2O. The reaction mixture was incubated at 37°C for 2 hrs, and the DNA template was removed by addition of 2 µl DNaseI (Fermentas) and the following incubation at 37°C for 30 min. Antisense RNA probe was purified with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and eluted with 30 µl RNase-free H_2O. The purified RNA probe was stored at -20°C and diluted in hybridization mix to a final concentration of 1 mg/ml for *in situ* hybridization.

3.1.10 Extraction of the total RNA from staged embryos and animal caps

2-4 embryos or 10-15 animal caps were collected in an eppendorf tube and immersed with 400 µl Trizol. After vortex for 3 min, the embryos and animal caps were completely disrupted with a fine syringe. The lysate was centrifuged at room temperature for 5 min. The supernatant was transferred to a new tube and then added with 0.2 volume of chloroform. This two-phase mix was mixed for 30 sec and centrifuged at 4°C for 10 min. The aqueous supernatant (around 200 µl) was transferred to a new tube and re-extracted with an equal volume of chloroform (mix for 30 sec followed by centrifugation at 4°C for 5 min). The supernatant was transferred to a new tube, mixed with an equal volume of isopropanol, and left to stand at -20°C for 30 min. The precipitated RNA was isolated by centrifugation at maximal speed at 4°C for 30 min. After washing with 400 µl 70% ethanol and air-drying, the pellet was resuspended in RNase free H_2O (20-30 µl). Genomic DNA was removed by adding 1 µl DNase I (Fermentas) and the following incubation at 37°C for 30 min.

3.1.11 Reverse transcriptase-polymerase chain reactions (RT-PCR)

The first strand cDNA was synthesized with the SuperScript™ II Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's protocol. 200 ng of total RNA from staged embryos, or animal caps was mixed with 1 µl random hexamer primer (0.2 µg/µl) and filled with H_2O to a volume of 12 µl. After gently mixing, the mixture was incubated at 70°C for 10 min, than the mixture was cooled down to room temperature. To the mixture were further 8 µl 5x transcription buffer, 4 µl 0.1M DTT, 1 µl Ribonuclease Inhibitor (20 u/µl), 4 µl 10 mM dNTP mix and 2 µl Reverse Transcriptase (200 u/µl) added. This mixture with a final volume of 40 µl was incubated at 42°C for 90 min followed by heating to 70°C for 10 min to stop the reaction. A standard 50 µl PCR reaction contained 1 µl cDNA obtained from RT reaction, 5 µl of 10x reaction buffer, 10 µl 5x Master mix (supplied with Taq polymerase), 1 µl of 10 mM

dNTP mix, 0.5 µl of specific primer mixture (forward and reverse primers, 7.5 µM for each), 0.3 µl Taq polymerase (5 U/µl, Fermentas) and 31.7 µl ddH_2O. Several forward and reverse primers were used. PCR program used are shown as follows: pre-denaturation at 94ºC for 2 min, 26 or 28 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC or 58ºC for 30 sec and extension at 68 ºC for 2 min, followed by a final extension at 72ºC for 10 min. The PCR products were separated on a 1.7% agarose gel and imaged with Bio-Rad Gel Doc 2000 (Bio-Rad, USA).

3.2 *In vitro* transcription-translation assay

In vitro transcription-translation assay was used to analyze the ability of ER71-GR and ER71-Myc-GR constructs to form a protein. It was performed in a 12.5 µl reaction with the TnT®-Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's user manual. The reaction mixture contained 6.25 µl TnT® Rabbit reticulocyte lysate, 0.5 µl TnT® Reaction buffer, 0.25 µl amino acid mixture (1 mM, minus Methionine), 0.25 µl RNase OUT™ ribonuclease inhibitor (40 u/µl, Invitrogen), 200 ng of ER71-GR circular plasmid or ER71-Myc-GR plasmid as the template, TnT® SP6 RNA Polymerase, 0.5 µl L-[^{35}S] Methionine (1,000 Ci/mmol at 10 mCi/ml) and appropriate amount of DEPC-H_2O to fill to a final volume of 12.5 µl. The reaction mixture was incubated at 30°C for 1.5 hr. After the incubation, an equal volume of 2x SDS gel loading buffer was mixed with the reaction mixture and heated at 95°C for 5 min. Proteins generated from the *in vitro* transcription-translation reaction were then analyzed on a 12% polyacrylamide gel marked with a Prestained Protein Ladder (Fermentas). The gel was run at 30mA, 200V through the starking gel, and then run at 50mA, 200V. After electrophoresis, the gel was dried then exposed on a Kodak BioMax XAR film (Kodak) in a Kodak X Omatic cassette (Kodak) overnight. On the next day, the film was developed and the proteins with different molecular weight could be visualized.

3.3 Handling and manipulation of *Xenopus* embryos

3.3.1 Preparation of embryos from *Xenopus laevis*

One day before egg collection, female albino and pigmented *Xenopus laevis* frogs was primed with 50-100 U of human chorionic gonadotropin (HCG). For induction of full ovulation, 500-1000 U HCG was injected into the dorsal lymph sac of frogs 10 hrs prior to egg collection. Eggs were fertilized *in vitro* with minced testes in 0.1x MBS, dejellyed with 2% cystein hydrochloride (2% L-cystein hydrochloride, pH 7.8-8.0), and cultured in 0.1x MBS. Albino embryos were stained with Nile blue solution after dejellying. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

3.3.2 Microinjection

The microinjection needles were prepared with borosilicate glass capillaries (Harvard apparatus, UK) using the Narishige PN-30 needle puller (Narishige, Japan). The needles were back-filled using microloaders (Eppendorf). Prior to microinjection, embryos were transferred to 1x MBS and then arranged on a glass slide with little buffer left. The injection was performed with a pneumatic PicoPump PV820 injector (Helmut Saur Laborbedarf, Germany) on a cooling plate. A volume of 5 nl mixture of desired synthetic RNA with the synthetic β-gal RNA was injected in a dorso-animal blastomere of embryos at the 2-cell stage. After injection, the embryos were cultivated in 1x MBS in Petri dishes for 1 hr and then in 0.1x MBS till the desired stages. Embryos were fixed in MEMFA at the desired developmental stage for 30 min. After washing three times for 10 min in PBS, embryos were transferred to X-Gal staining solution until staining was sufficient. Afterwards, the embryos were re-fixed in MEMFA for 1.5 hr. For whole-mount *in situ* hybridization assay, embryos were dehydrated with absolute ethanol and stored at -20°C.

3.3.3 Preparation and cultivation of the animal tissue explants (animal caps)

The synthetic mRNA was injected into both blastomeres of the *Xenopus laevis* animal pole embryos at 2-cell stage. After the embryos reaching the blastula stage (NF St. 9-9.5) the animal caps were dissected in 0.5x MBS using a gastromaster equipped with a red microsurgery tip of 400 µm. 15-20 caps were cultured in petri dishes coated with 1% agarose in 0.5x MBS, and incubated until they reached NF St. 14-16 with and without dexamethasone (500x) at 16-18 °C, finally the animal caps were collected for *in situ* or RNA extraction (RT-PCR).

3.4 Analysis Methods

3.4.1 Whole-mount *in situ* hybridization (WMISH)

The whole-mount *in situ* hybridization was performed according to a three days procedure as described previously (Hollemann et al., 1998a).

Day 1:

Embryos were rehydrated through the ethanol series (75%, 50% in dH_2O and 25% in PTw) for 5 min in each step, followed by the intensive 4 times washing with PTw for 5 min. Embryos were then digested with 10 µg/ml Proteinase K (Sigma) in PTw at room temperature for 10-20 min according to the stage of the embryos. Subsequently, embryos were washed twice with 0.1 M triethanolamine (pH 7.5) for 5 min and acetylated by two additions of 12.5 µl acetic anhydrite into the 5 ml embryos incubation tube fully-filled with 0.1 M triethanolamine (pH 7.5) and incubated at room temperature for 5 min after each addition. After twice washing with PTw for

5 min, embryos were re-fixed with PFA at room temperature for 20 min, afterwards, embryos were washed 5 times with PTw for 5 min and rinsed with 1 ml mixture of equal volumes of PTw and hybridization mix. After a preincubation in 500 µl hybridization mix at 65ºC for 10 min, the embryos were pre-hybridized in 1 ml hybridization mix at 60ºC for 6 hrs. Embryos were then hybridized overnight in 1 ml hybridization solution containing the appropriate amount of antisense probe at 60ºC.

Day 2:

The probe/hybridization mix was recovered and stored at -20°C for reuse. The embryos were refilled with 1 ml hybridization mix and incubated at 60ºC for 10 min, followed by 3 times washing with 2x SSC at 60ºC for 15 min each time. Unspecific bound antisense probe was digested by an RNase Mix (20 µg/ml RNase A, 10 U/ml RNase T1 in 2x SSC) at 37ºC for 60 min. Embryos were washed once with 2x SSC for 10 min at room temperature and then twice with 0.2x SSC at 60ºC for 30 min. The procedure afterward was performed under ambient temperature except specified. After washing twice with MAB for 15 min, embryos were blocked in MAB/BMB for 20 min and then in MAB/BMB/HS for 60 min. Embryos were incubated in MAB/BMB/HS containing 1:5000 diluted anti-Digoxigenin/AP (Roche) for 4 hrs. After incubation, embryos were washed 3 times with MAB for 10 min and then overnight at 4ºC.

Day 3:

Embryos were washed 5 times with MAB for 5 min and then equilibrated twice in the chilled APB for 10 min. After transferring to a pre-cooled color reaction solution (APB containing NBT and BCIP), embryos were incubated on ice in the dark until sufficient staining was reached. The staining reaction was stopped by directly changing the staining solution to ethanol. The following twice replacement of fresh ethanol helped to reduce the background. Embryos were rehydrated through an ethanol series (75%, 50% and 25% ethanol) for 5 min in each step and stored in MEMFA at 4ºC.

3.5 Histological Method

Vibratome section

Specimens of embryos after whole-mount *in situ* hybridization were transferred to PBS and then infiltrated in gelatin-albumin solution for 20 min. 1.5 ml gelatin-albumin was mixed with 105 µl 25% glutaraldehyde on ice quickly and poured into a plastic mold (Polyscience) to make the lower layer. The infiltrated embryos were later transferred on the solidified gelatin-albumin layer. After the solution around the embryos was carefully removed, the upper layer was prepared as the lower layer and filled over the embryos. Sections (30 µm) were cut on a Leica VT1000S vibratome (Leica, Germany) as described previously (Hollemann et al., 1999).

3.6 Cell biological methods

3.6.1 Cell Culture material, media and solution

Cell culture flasks (25 and 75cm^2) and the 12-well flat plates were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany). Dulbecco's MEM (DMEM) was purchased from PAA Laboratories GmbH (Cölbe, Germany).

3.6.2 Transfection of ER71-Myc-GR plasmid DNA into HeLa cells

HeLa cells are a cell line which was obtained from cervical carcinoma (Gey et al., 1952). The cells were cultured in 75cm^2 flask and treated with trypsin then transferred into a 12 well plate at a density of 2.5x 10^6 cell/well in 1 ml of cell culture medium and incubated at 37 ºC, 5 % CO_2 for transfection experiment. The cells were incubated with serum free medium 1 hr before transfection, then 4 µl Lipofectamine 2000 (Invitrogen, Deutschland) was mixed with 96 µl Opti-MEM by pipetting and incubated at room temperature for 5 min. 100 µl of the mixture was added per well after incubation with 1.5 µg (ER71-Myc-GR) plasmid for 20 min at room temperature. Afterwards, the cells were incubated for 4 hrs at 37˚C. Then the cells were incubated with and without dexamethasone (500x) in cell culture medium containing serum overnight at 37 ˚C, 5% CO_2, finally the cells were fixed for immunofluorescence staining.

3.6.3 Immunofluorescent staining of HeLa cells

HeLa cells were plated one day prior to staining in order to achieve 60, 80% confluency. After the medium was removed, the cells were washed with 1xPBS twice for 5 min and fixed with 4% formaldehyde for 30 min at room temperature. Then the cells were washed gently twice in 1xPBS for 5 min and covered with permeabilization solution for 5 min. Again, they were washed with 1xPBS for 5 min. Afterwards the cells were covered with blocking solution for 30 min at room temperature. After the primary antibody (α Myc-mouse) was diluted 1:400 in blocking reagent and overlaid the cells for 2 hrs at 37ºC. To limit evaporation, a piece of Parafilm was used to tightly cover the opening of the chamber slide. The cells were washed with 1xPBS, 3 times for 5 min and the blocking solution was added for 30 min. The secondary antibody (α-goat-Alexa 488) was diluted 1:800 in antibody buffer, then overlaid onto HeLa cells, and incubated for 45 min at 37ºC. The cells were washed with 1xPBS twice, 5 min with ddH_2O and with 100 % Ethanol twice. Finally, the cells were stained with 20 µl of Mowiol/DAPI (250 µl Mowiol and 1.25 µl DAPI). The fluorescence images were taken with a Nikon Eclipse E600 Fluorescence microscope using an Apo TIRF oil immersion objective.

4. Results

4.1 Cloning of two inducible *Xenopus* ER71 constructs, Myc-ER71-GR and ER71-GR

Recently, it was reported that overexpression of *Xenopus* ER71-mRNA at early stages of embryogenesis interfered with gastrulation (Neuhaus et al., 2010), making it difficult to analyze the effect of ER71 overexpression during early and late stages of vascular development. To overcome this problem I made constructs to express inducible ER71-fusion proteins. In one construct, the complete ER71 ORF was fused in-frame to human glucocorticoid receptor ligand-binding domain (GR). In a second construct, a Myc-tag was added in frame to the N-terminus of this ER71-GR construct, resulting in a Myc-ER71-GR construct (Figure 4.1 A). When the ER71-GR fusion proteins are expressed in a cell and glucocorticoid receptor ligands are absent, the GR-fusion protein binds to hsp90 (Heat-shock protein 90) and therefore is retained in the cytoplasm. When a ligand is present, it competes with hsp90 for binding to the GR and releases the GR-fusion protein from hsp90. Consequently, the GR-fusion protein, in this case, Myc-ER71-GR or ER71-GR, could enter the nucleus and induce the transcription of its target sequences (Figure 4.1 B) (Gammill and Sive, 1997). Induction of ER71 function could be achieved by adding a synthetic ligand, such as dexamethasone (Dex) to the buffer containing the injected embryos at desired stages. To confirm the successful expression of these two constructs, an *in vitro* transcription and translation assay was performed using the TnT®-Coupled Reticulocyte Lysate system. The translated proteins showed their expected molecular weights (Figure 4.1 D).

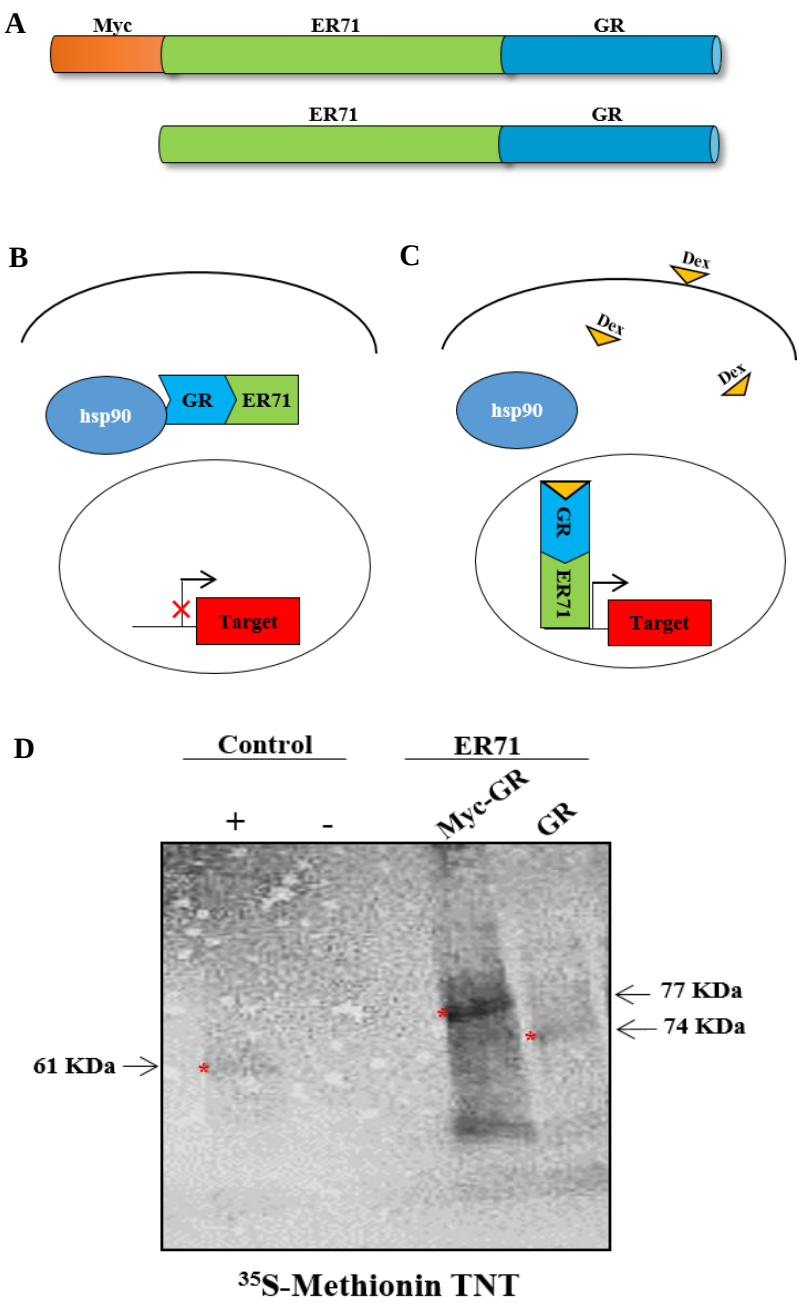


Figure 4.1 The schematic diagrams of the structures and working mechanism of inducible Myc-ER71-GR and ER71-GR constructs. (A) Myc-ER71-GR and ER71-GR constructed by in-frame fusion of ER71 ORF and glucocorticoid receptor ligand binding domain (GR), in both constructs. Myc-Tag was added in-frame to Myc-ER71-GR construct. (B) In the absence of the glucocorticoid receptor ligand, Dexamethasone (Dex) (left), ER71-GR binds to hsp90 in the cytoplasm. (C) With the presence of Dex (right), ER71-GR is released from hsp90 and enters the nucleus, where it can activate target genes. (D) To test whether the constructs used for the overexpressed studies are functional, a coupled Transcription and translation assay (TNT®-coupled reticulocyte lysate system) was performed. After *in vitro* transcription and translation of Myc-ER71-GR (77 kDa) and ER71-GR (73 kDa), proteins of the expected size could be detected. As a positive control a luciferase plasmid (61 kDa) was used. In a negative control reaction no plasmid DNA was added (– control). The translation mixtures were separated by SDS-PAGE 12% and the autoradiographically labeled products was detected by exposure to X-ray films. Glucocorticoid Receptor Ligand Binding Domain, (GR LBD, Short GR). Heat shock protein 90 (hsp90), Dexamethasone (Dex).

4.2 The translocation of Myc-ER71-GR protein from the cytosol to the nucleus in HeLa cells is induced by dexamethasone treatment

To test the inducible ER71-GR construct *in vivo* a transfection experiment was performed using HeLa cells. The cells were transfected with (1.5 µg) Myc-ER71-GR plasmid, and incubated overnight without and with dexamethasone. Then the cells were fixed and the fusion protein (Myc-ER71-GR) was detected with an Anti-Myc Tag antibody. In the cells which were incubated without dexamethasone (-Dex), we could see that most of Myc-ER71-GR protein was retained in the cytoplasm. However, a small fraction was found in the nucleus, most likely due to the high level of expression. Whereas in cells, which were incubated with dexamethasone (+Dex), the Myc-ER71-GR protein translocated from the cytosol to the nucleus (Figure 4.2 B). This result indicates that the cellular localization of inducible ER71 proteins was mostly dependent on dexamethasone addition in cell culture.

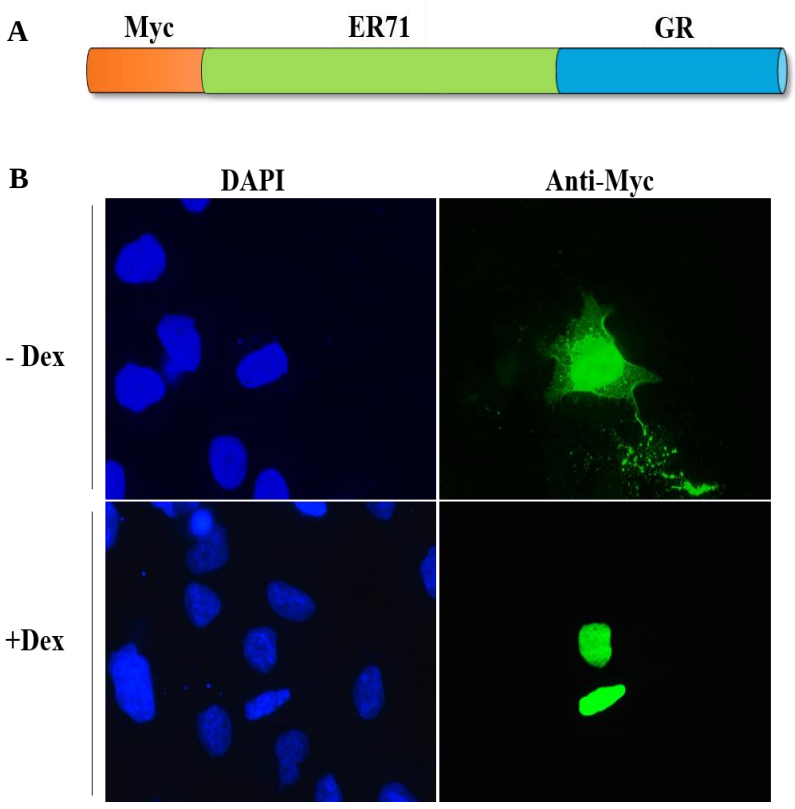
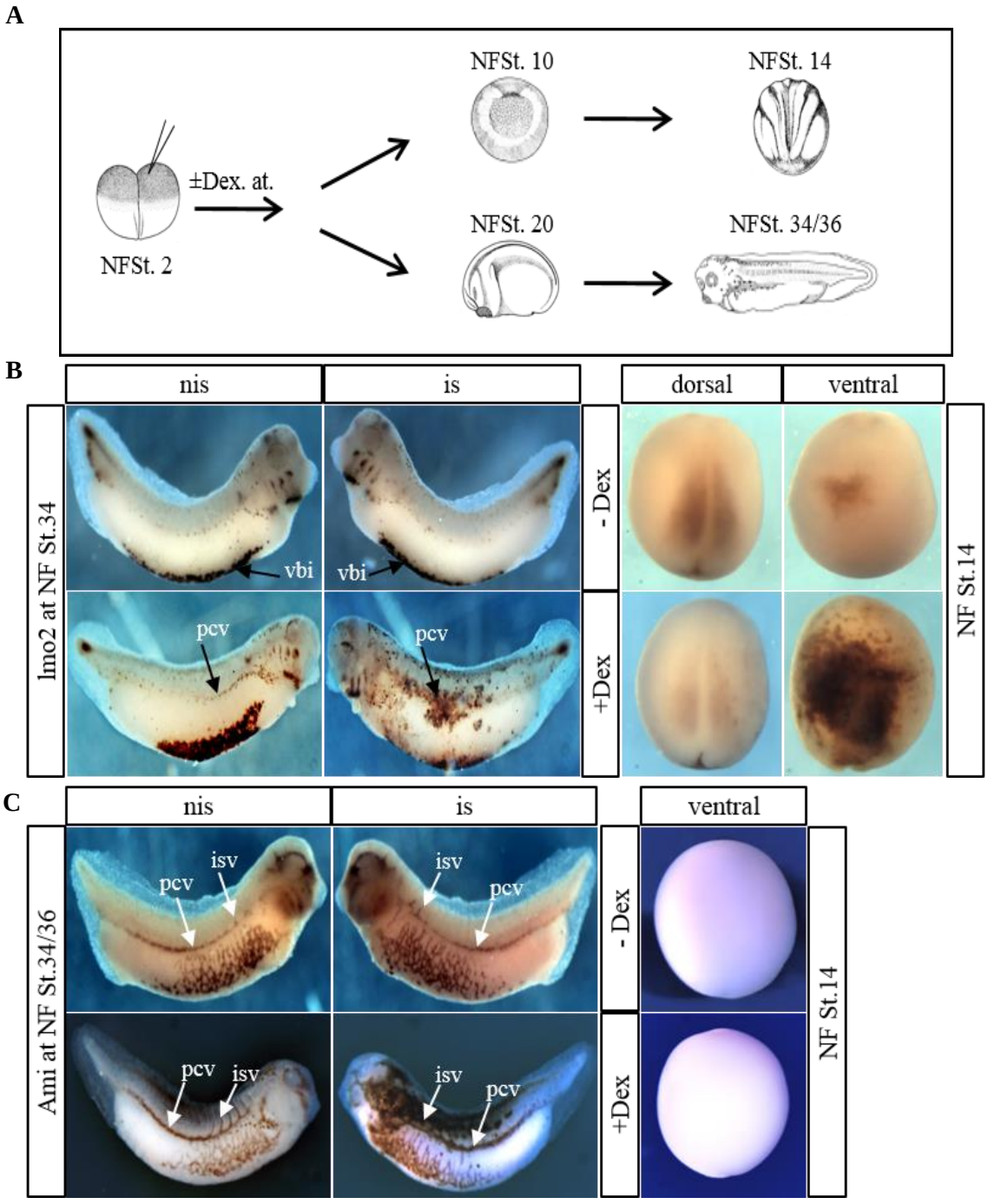


Figure 4.2 The localization of *Xenopus* Myc-ER71-GR protein in transfected HeLa cells. (A) Schematic diagram of *Xenopus* Myc-ER71-GR. (B) Myc-ER71-GR DNA (1.5 µg) was transfected to HeLa cells and the cells were incubated without (-Dex) and with (+Dex) Dexamethasone. Then the cells were fixed for immunostaining using DAPI to stain the cell nuclei and Anti-Myc Tag antibody to stain the Myc-ER71-GR protein. In cells which were incubated without dex (-Dex) Myc-ER71-GR protein was located in the cytosol, while in presence of dex (+Dex) all Myc-ER71-GR protein was translocated into the nucleus.

4.3 The analysis of ER71-GR overexpression at early and late embryonic stages

In this experiment, we asked whether ER71-GR could induce the ectopic expression of vascular and hematopoietic marker genes at early as well as later stages of *Xenopus* development. To answer this question, an overexpression experiment with ER71-GR was performed. ER71-GR mRNA (200 pg) and β-gal RNA were co-injected in one of the blastomeres at 2-cell stage. When the injected embryos reached NF St. 10 or 20, dexamethasone was added to the buffer. After further incubation the embryos were collected at NF St. 14 or 34/36 and used for WMISH to analyze the expression of hematopoietic marker (*lmo2*) and vascular marker (*ami*). In embryos that were incubated without dexamethasone the expression pattern of lmo2 and Ami at the injected side was indistinguishable from the pattern at the non-injected side (Figure 4.3 B, C). When dexamethasone was added to the embryos (+Dex) at NF St. 10, most of the embryos had gastrulation defects and did not survive, as was reported in Neuhaus et al., 2010. However, when embryos were analyzed at NF St. 14, we could observe ectopic expression of lmo2 in the dorsal and ventral region of the embryos (40%). But when dexamethasone was added to the embryos (+Dex) at NF St. 20 and collected at NF St. 34, the embryos developed normal showing massive overexpression of lmo2 at the injected side (50%), which was mainly restricted to the region around the posterior cardinal vein (Figure 4.3 B, D). When dexamethasone was added to the embryos (+Dex) at NF St. 10 and analyzed at NF St. 14, we could not observe expression of the marker gene *ami*. While**,** when dexamethasone was added to the embryos (+Dex) at NF St. 20 and collected at NF St. 34/36, we could observe massive overexpression of Ami at the injected side (63%), which was mainly restricted to the region around the intersomitic vein and the posterior cardinal vein (Figure 4.3 C, D).

These data indicated that ER71-GR could induce ectopic expression of the hematopoietic marker lmo2 at early embryonic stages (NF St. 14) as well as at later stages (NF St. 34). In contrast, the vascular marker *ami* could only be induced at later stages (34/36) of *Xenopus* embryogenesis.


A
NFSt. 10
NFSt. 14
±Dex. at.
NFSt. 2
NFSt. 20
NFSt. 34/36
B
nis
is
dorsal
ventral
lmo2 at NF St.34
- Dex
+Dex
NF St.14
vbi
vbi
pcv
pcv
C
nis
is
ventral
Ami at NF St.34/36
- Dex
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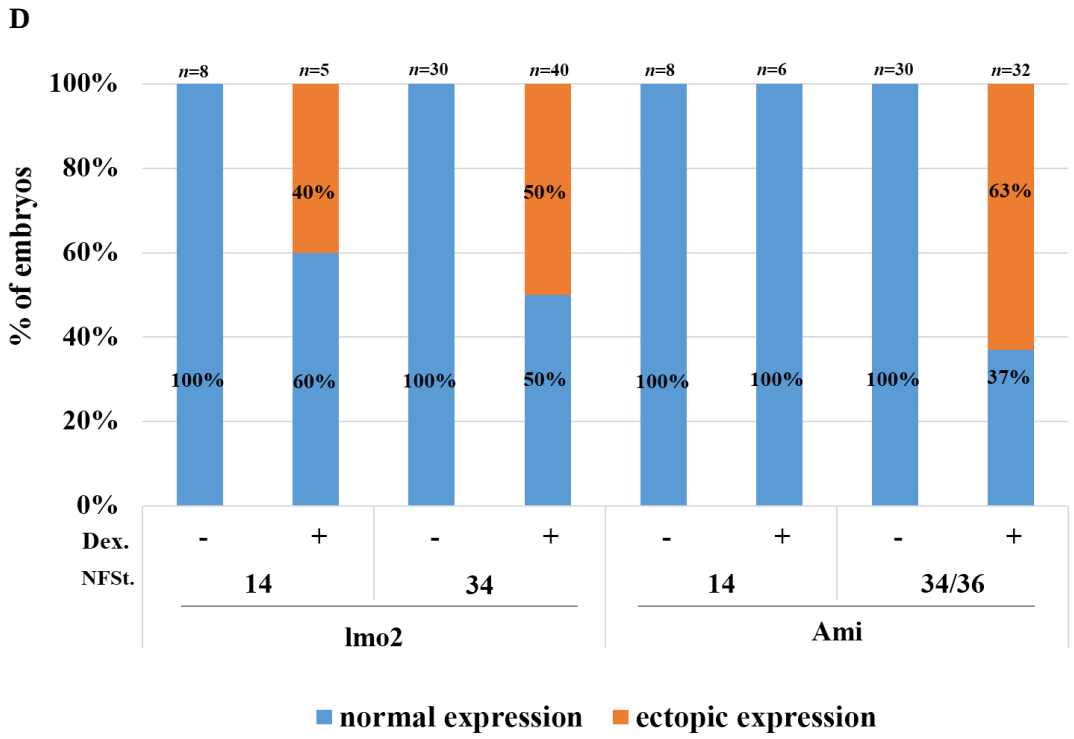

Figure 4.3 ER71-GR induced the ectopic expression of hematopoietic (*lmo2*) and vascular (*ami*) markers in *Xenopuos* embryos. (A) Experimental scheme. Albino embryos were co-injected with ER71-GR mRNA (200 pg) and β-gal RNA in one of the blastomeres at 2-cell stage, then embryos from NF St. 10 or 20 onwards were incubated without and with Dex until they reached developmental stages equivalent to NF St. 14 and 34/36 stages. (B, C) WMISH anylysis of treated embryos to analyze the expression of lmo2 and Ami. The non-injected side (nis) and the injected side (is) of the embryos are shown. (B) The embryos which were incubated without Dex and collected at NF St. 14 or 34 showed the normal expression of lmo2 in both sides. But the embryos which were incubated with Dex and collected at NF St. 14 and NF St. 34 showed ectopic expression of lmo2 in the injected side. (C) The ectopic expression of Ami was detected only at NF St. 36 in the injected side. (D) Analysis of ER71-GR injected embryos examined for the expression of lmo2 and Ami at NF stage 14 and 34/36, when induced at NF stage 10 and 20 respectively.vbi, ventral blood islands; pcv, posterior cardinal vein; isv, intersomitic veins. *n*, the total number of collected embryos.

4.4 Response to ER71 signaling at different stages of embryonic development.

In the experiments described in chapter 4.3, we had shown that the expression of the hematopoietic marker gene *lmo2* could be induced at earlier developmental stages by ER71, than the expression of the vascular marker gene *ami*. To analyze the responsiveness of hematopoietic and vascular markers in more detail, we made a series of experiments in which the translocation of overexpressed ER71-GR to the nucleus was induced at consecutive time points.

ER71-GR mRNA (200 pg) were co-injected with β-gal into one of the blastomeres of a 2-cell stage embryo. The embryos were incubated until they reached stage 34/36. Dexamethasone was added at stage 12, 14, 18 or 20. ER71-GR injected embryos, which were incubated without dexamethasone (-Dex), showed normal expression of the vascular marker (*ami*) and the hematopoietic marker (*mpo*), (Figure 4.4 B; a, a', a'' and Figure 4.5 f, f', f'', respectively). However, the treatment of the embryos with dexamethasone starting at different stages (12, 14, 18 and 20) always induced the ectopic expressions of both makers *ami* (Figure 4.4 b', b'', c', c'', d', d'', e', e'') and *mpo* (Figure 4.5 g', g'', h', h'', i', i'', j', j'') at the injected side of the embryos.

These data showed that there were cells in the treated *Xenopus* embryos that could response to ER71-GR signaling during all stages analyzed, from late gastrulation stages (NF St. 12), to early (NF St. 14) and later neural stages (NF St. 18, 20). Interestingly, we could observe correlation between the extent of ectopic marker expression and the time point when dexamethasone was added. (Figure 4.6 B). To quantify the extent of ectopic expression we sorted the embryos into three groups: embryos showing no areas of ectopic marker gene expression, embryos showing moderate areas of ectopic marker gene expression and embryos showing large areas of ectopic marker gene expression. For both marker genes analyzed, we could observe that the number of embryos showing no ectopic expression decreased the later dexamethasone was added and the number of embryos showing moderate to strong observation increased.

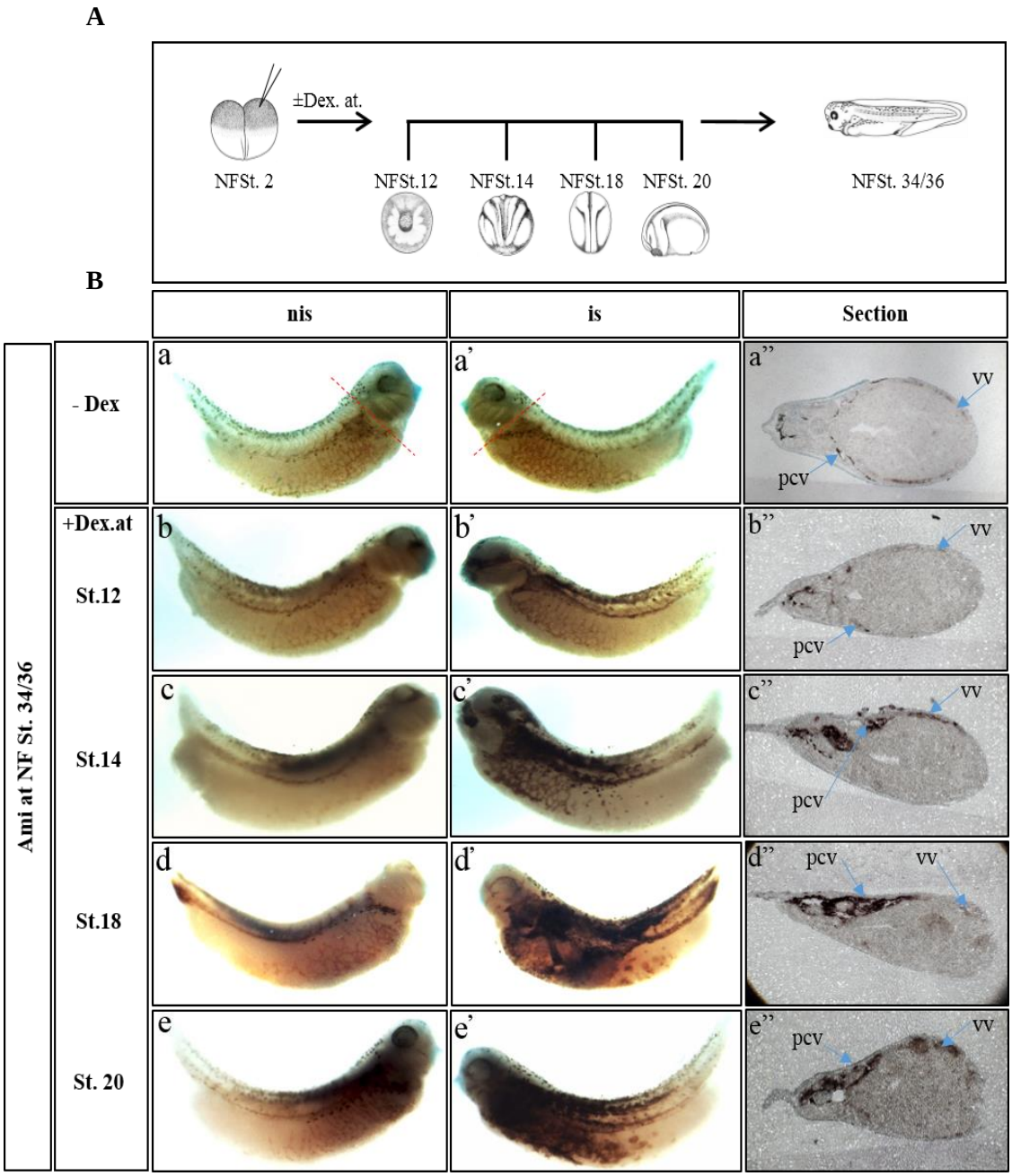

Figure 4.4 The effect of ER71-GR nuclear translocation on the expression of vascular marker (*ami*) at different embryonic stages. (A) experimental scheme. Albino embryos were injected with 200 pg ER71-GR mRNA in one of the blastomeres at 2-cell stage. When embryos reached NF St. (12, 14, 18 and 20) incubation with Dex was started until they reached developmental stages equivalent to NF St. 34 or 36. (B) WMISH analysis of expression of the vascular marker *ami*. (a, a', a''). Embryos incubated without Dex showed normal expression of Ami in both sides (nis, is). (a, b, c, d, e) Embryos incubated with Dex at NF St. (12, 14, 18 and 20) in the non-injected side ("nis" left side) showed also normal expression, but in the injected side ("is" right side) embryos (b', c', d', e', f') showed ectopic expression of Ami. (a'', b'', c'', d'', e'') Transversal sections of each embryo at the level indicated by red dashes. vv; viteline vein, pcv, posterior cardinal vein.

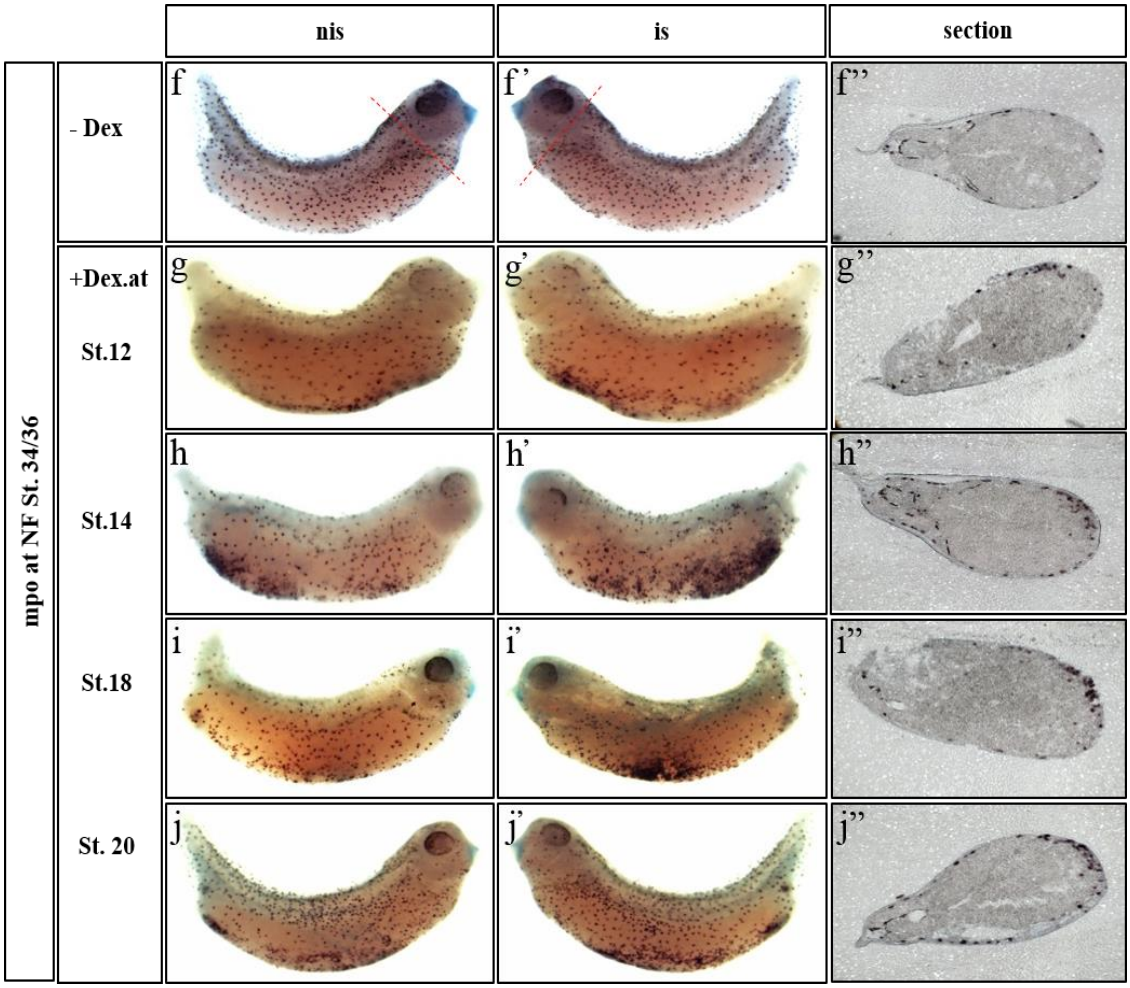

Figure 4.5. The effect of ER71-GR nuclear translocation on the expression of hematopoietic marker (*mpo*) at different embryonic stages. WMISH analysis of expression of the hematopoietic marker mpo. (f, f') Embryos incubated without Dex showed a normal expression of mpo in both sides (nis, is). (g, h, i, j) Embryos incubated with Dex at NF St. (12, 14, 18 and 20) in the non-injected side ("nis" left side) showed also normal expression but in the injected side ("is" right side) embryos (g', h', i', j') showed ectopic expression of mpo. (f'', g'', h'', i'', j'') Transversal sections of each embryo at the level indicated by red dashes. vv; viteline vein, pcv, posterior cardinal vein.

A

Ami				
The condations	**Total number of embryos**	**No ectopic expression**	**Weak ectopic expression**	**Strong ectopic expression**
- Dex	90	87	3	0
+ Dex at NFst.12	151	118	28	5
+ Dex at NFst.14	141	108	21	12
+ Dex at NFst.18	109	67	16	26
+ Dex at NFst.20	120	44	43	33
mpo				
- Dex	219	197	12	10
+ Dex at NFst.12	221	143	47	31
+ Dex at NFst.14	273	197	43	33
+ Dex at NFst.18	218	100	57	61
+ Dex at NFst.20	213	81	57	75

B

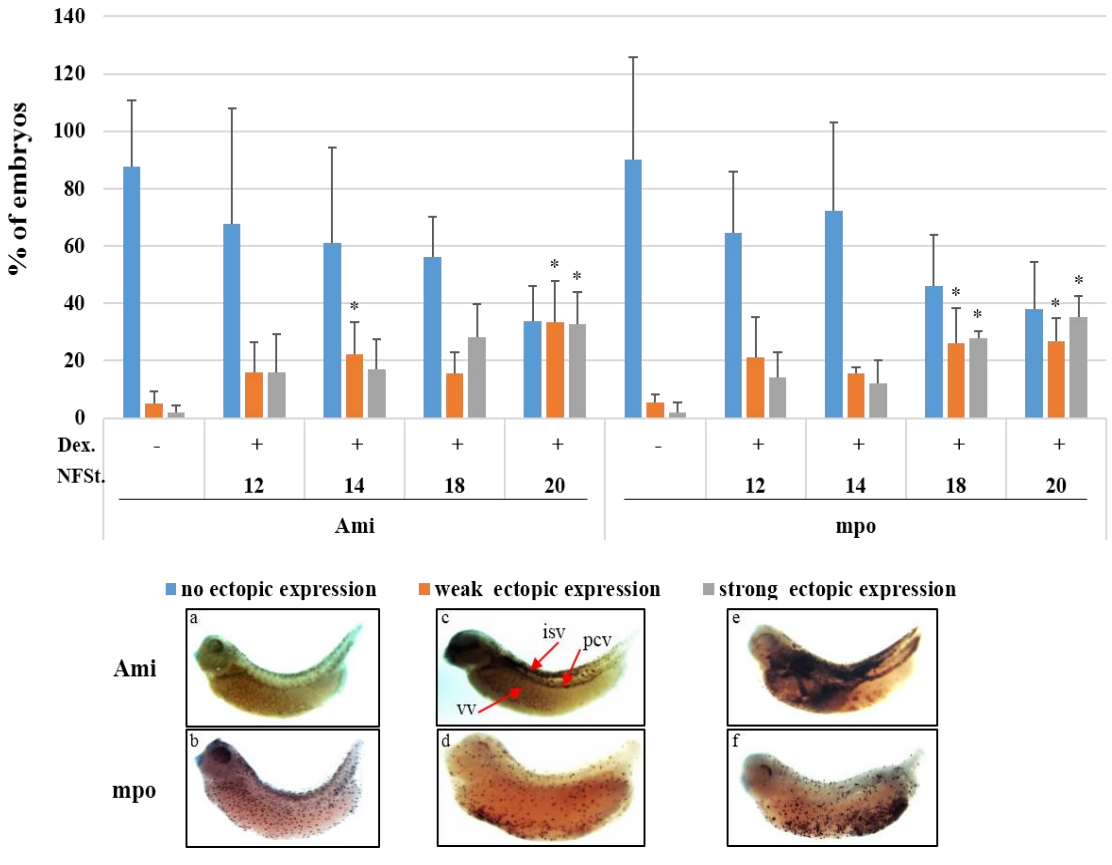

Figure 4.6 Quantification of ectopic expression of vascular and hematopoietic markers in *Xenopus* embryos injected with ER71-GR. (A) The number of embryos which show ectopic expression of the vascular (*ami*) and hematopoietic (*mpo*) markers. The ectopic expression of the vascular (*ami*) and hematopoietic (*mpo*) markers in *Xenopus* embryos was quantified, by sorting embryos into three groups, embryos with no ectopic expression, embryos with weak ectopic expression and embryos with strong ectopic expression. (B) Statistical analysis of ER71-GR injected embryos with ectopically expressed Ami and mpo. The blue bars represent embryos (a, b) showing no ectopic expression (the normal expression of

Ami, mpo) and the orange and grey bars represent embryos (c, d; e, f) showing week or strong ectopic expression, respectively. The percentage of embryos showing strong ectopic expresssion incresed when Dex was added at later stages. Values are given as means ± s.e.m. *, $p<0.05$, compared with embryos without Dex (-Dex). vv; viteline vein, pcv, posterior cardinal vein. Number of independent experiments = 3.

4.5 The translocation of Myc-ER71-GR protein from the cytosol to the nucleus in animal caps is dependent on dexamethasone treatment.

The animal cap consists of the ectodermal roof of a blastula stage embryo that can be excised and kept in culture under simplest conditions (Hollemann et al., 1998b). The cells of the animal cap are considered to have stem cell character and therefore are capable to respond to a wide variety of extra- and intra cellular stimuli, differentiating into endodermal, mesodermal and ectodermal cell types.

In the previous section it was revealed that released ER71-GR induced the expression of the vascular marker *ami* and the hematopoietic markers *lmo2* and *mpo* in *Xenopus* embryos. In line with these results, we wanted to identify novel target genes of ER71 at early and later stages of *Xenopus* development. Since the activity of ER71 at earlier stages of embryogenesis caused gastrulation problems and the subsequent death of the embryos, we choose the animal cap system. To establish conditions at which we could distinguish between background transcription and transcriptional upregulation caused by induced translocation of ER71-GR from the cytosol to the nucleus.

We injected various amounts of Myc-ER71-GR mRNA into both blastomeres of a 2-cell stage embryo and incubated them until they reached NF St. 9. At this stage, animal caps were dissected and then incubated without or with dexamethasone until they reached stages equivalent to stage 14 or 26. ER71 protein localization in the animal caps was analyzed by immunostaining using anti-Myc antibody. When injecting 200 pg of synthetic Myc-ER71-GR mRNA we could observe solid expression of Myc-ER71-GR protein. When animal caps were incubated without dexamethasone (-Dex), Myc-ER71-GR protein was retained in the cytosol of animal cap cells at both stages analyzed (NF St. 14, 26) (Figure 4.7 B; a, b, c). While in induced animal caps we could observed that Myc-ER71-GR protein was localized in the nucleus at NF St. 14 as well as NF St. 26 (Figure 4.7 B; d, e, f). This result showed that under appropriate conditions we could force the nuclear translocation of Myc-ER71-GR protein in animal caps, where it could induce the transcription of its target genes.

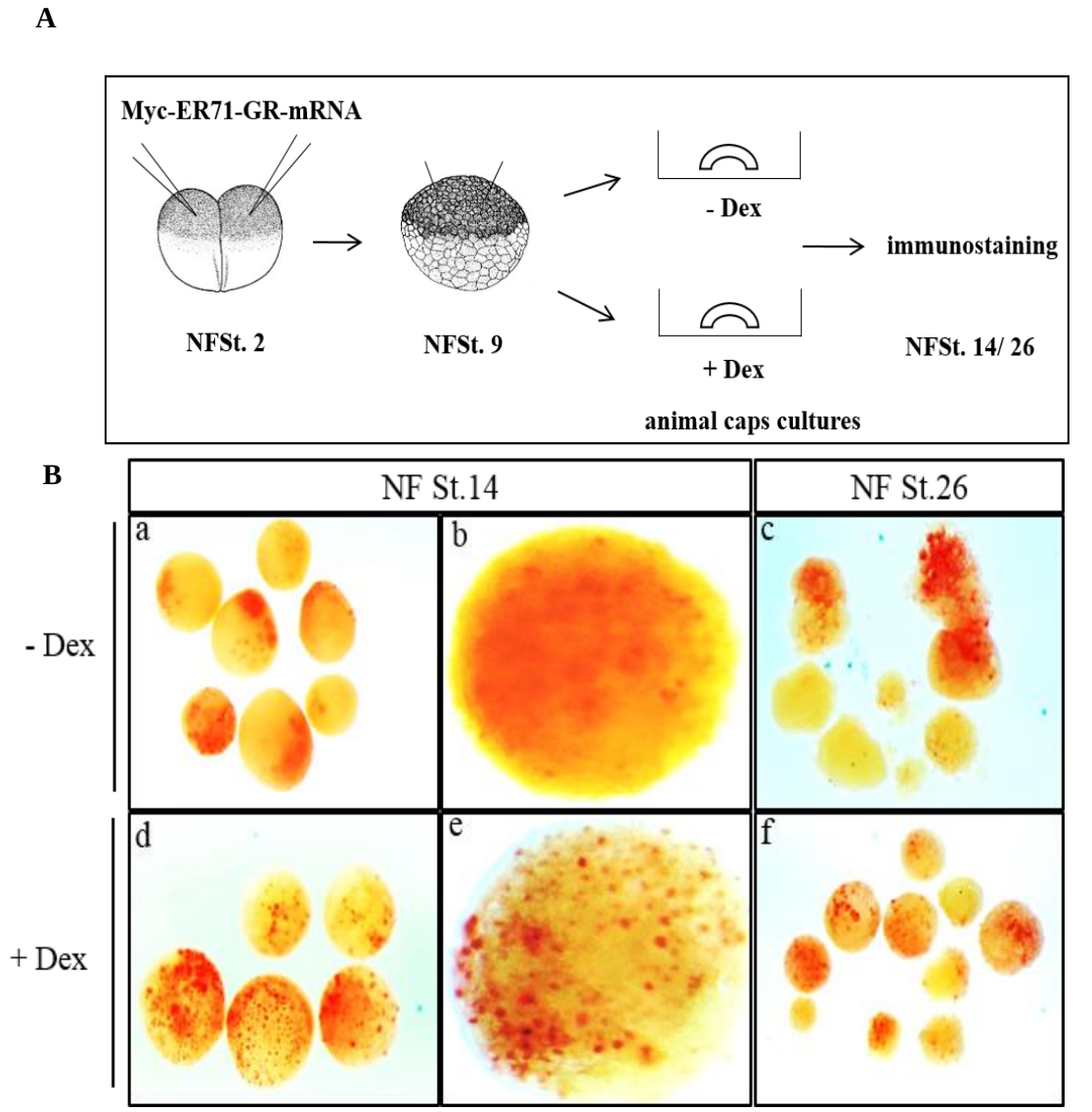

Figure 4.7 ER71 protein was translocated into the nucleus in treated animal caps. (A) Experimental scheme. Albino embryos were injected with 200 pg Myc-ER71-GR mRNA into both blastomeres of a 2-cell stage embryo. When the embryos reached NF St. 9, the animal caps were dissected and then incubated without and with Dex until they reached NF St. 14 or 26. Then they were collected for immunostaining. (B) Animal caps incubated without Dex until they reached NF St. 14 (a, b), and NF St 26 showed a diffuse distribution of Myc-ER71-GR protein in the cytosol. In dexamethasone treated animal caps at NF St. 14 (d, e) and at NF St. 26 (f) Myc-ER71-GR protein was located in the nucleus.

4.6 Overexpression of ER71-GR in dexamethasone treated animal caps induced the expression of endogenous ER71

In this experiment, we assayed the endogenous expression of ER71 gene in *Xenopus* embryos and animal caps in order to explore whether the endogenous expression of ER71 was down or upregulated by injection of ER71-GR mRNA. Total mRNA was extracted from different stages of wild type *Xenopus* embryos and from animal caps, which were injected into both blastomeres at the two-cell stage with 150 pg ER71-GR, then dissected at NF St. 9 and subsequently incubated without and with dexamethasone. The endogenous ER71 gene expression was monitored by a semi-quantitative (RT-PCR) in embryos and animal caps (Figure 4.8 A, B), using a primer pair located in the untranslated exon 1, upstream of the ATG sequence. In normally developing embryos, earliest endogenous expression of ER71 was detectable at late gastrulation stage (NF St. 12) and the level of ER71 expression increased until NF St. 27. Subsequently expression decreased again and was barely detectable at NF St. 42. In untreated animal caps, ER71 expression was not detectable. However, in dexamethasone treated animal caps, expression of endogenous ER71 was clearly detectable, indicating that endogenous expression of ER71 can be positively auto-regulated.

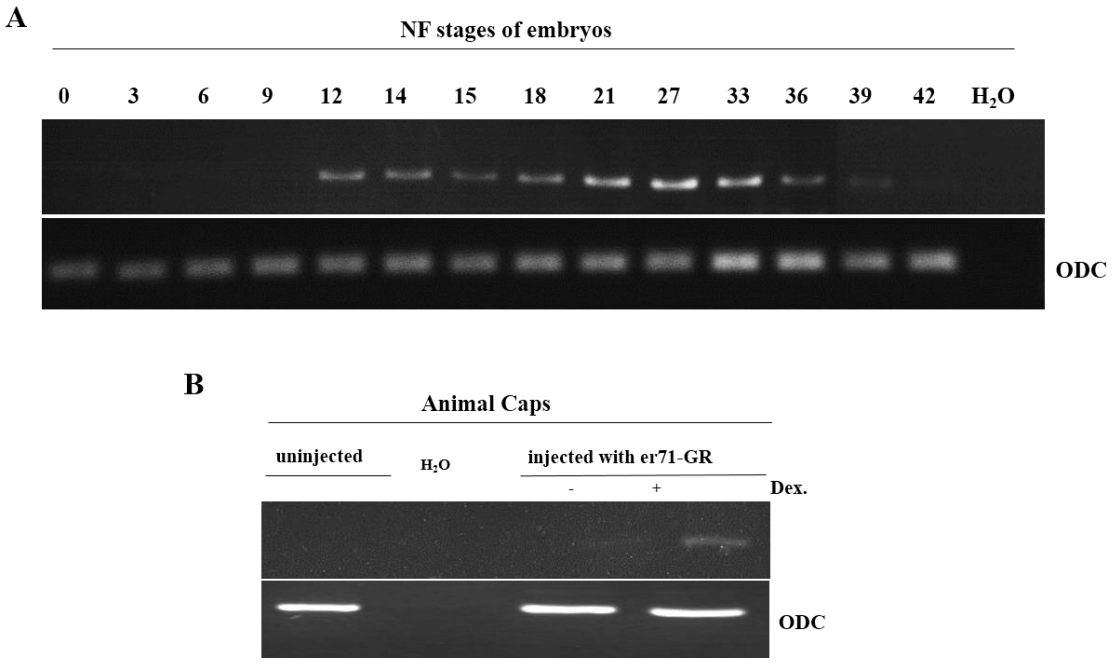

Figure 4.8 The endogenous level of ER71 in *Xenopus* embryos and ER71 overexpressing animal caps. (A) Analysis of endogenous expression level of ER71 in the wild type embryos by RT-PCR. Total RNA was extracted from wild type embryos at indicated stages. The expression level of Ornithine decarboxylase (ODC-1) examined in parallel as a control. (B) 150 pg of ER71-GR-mRNA was injected into both blastomeres at 2-cell stage, animal caps were dissected at NF St. 9, and the caps were incubated without and with Dex until they reached a time point equivalent to NF St. 14, were collected for RNA extraction and the RT-PCR was carried out for the analysis. As shown, the endogenous expression of ER71-GR was induced in treated animal caps (+Dex).

4.7 The non-homogeneity responses of animal cap cells to ER71-GR signaling

We had shown that ER71-GR could induce ectopic expression of vascular and hematopoietic markers in distinct regions of *Xenopus* embryos. Since animal cap structures represent an early pluripotent cellular origin of the entire embryo, we wanted to analyze whether all the cells of an animal cap respond homogenously to ER71 overexpression. To answer this question, the expression of hematopoietic marker *lmo2* was analyzed using WMISH. After ER71-GR was injected into both blastomeres at the 2-cell stage, animal caps were dissected and incubated without and with dexamethasone, and collected at NF St. 14. When animal caps were incubated without dexamethasone (-Dex) and collected at NF St. 14, 23.1% of the injected animal caps showed ectopic expression of lmo2. However, 75 % of animal caps incubated with dexamethasone (+Dex) and collected at NF St. 14, showed ectopic expression of lmo2 (Figure

4.9 B, C). These results indicate that ER71-GR could induce the ectopic expression of hematopoietic marker gene (*lmo2*) in treated animal caps, however, the response of animal caps cells to ER71-GR signaling was not homogeneous.

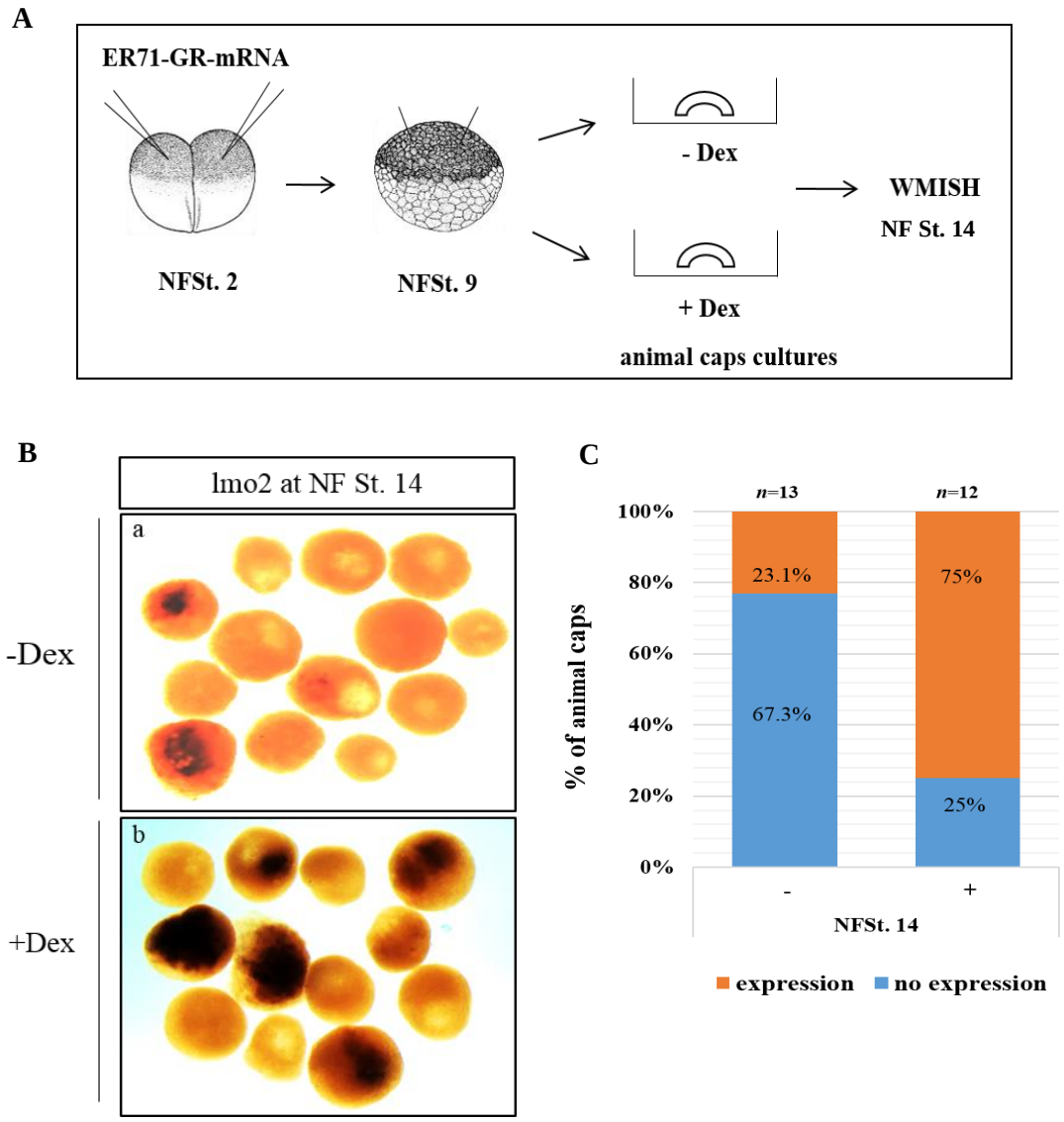

Figure 4.9 Induction of ER71-GR to the hematopoietic marker (*lmo2*) in animal caps. (A) Experimental scheme, embryos were injected with 200 pg ER71-GR mRNA in both blastomeres at 2-cell stage. When the embryos reached NF St. 9, the animal caps were dissected, and then incubated with and without Dex until they reached NF St. 14. They were collected for WMISH. (B) Animal caps were incubated with and without Dex until they reached NF St. 14 and were collected for WMISH analysis. (C) The percentage of animal caps which showed ectopic expresssion of lmo2. *n*, the total number of collected animal caps.

4.8 ER71-GR induces various vascular and hematopoietic markers in *Xenopus* animal caps

As described above, we have shown that ER71-GR injected into early *Xenopus* embryos induced the ectopic expression of vascular and hematopoietic markers (*ami, lmo2, mpo*). We had also shown that the ER71-GR system could be used in animal caps to direct temporal translocation of ER71 protein to the nucleus. Since we wanted to identify novel ER71 target genes, we made a series of experiments to collect well-defined RNA samples of corresponding animal caps. We dissected animal caps from embryos, which were injected with 150 pg of synthetic ER71-GR capped RNA into both blastomeres of a 2-cell stage embryo. Subsequently, animal caps were incubated without and with dexamethasone until they reached a developmental stage equivalent to NF St. 14, when they were used to isolate total RNA. To evaluate the efficiency of ER71 target gene induction, we subsequently analyzed the expression of a number of vascular and hematopoietic marker genes, which had been shown to be upregulated by ER71 overexpression previously. Using semi-quantitative RT-PCR, we could show that the expression of the vascular marker genes (*ve-cadherin, flk1, Fli, msr*) and the hematopoietic marker genes (*scl, mpo, lmo2*) were clearly upregulated in dexamethasone treated animal caps compared to untreated animal caps demonstrating that we prepared samples with distinguishable RNA content.

A number of biological samples of such animal cap experiments were analyzed using RT-PCR and the isolated RNA from three animal cap samples (1, 2, 3) were used for deep RNA sequencing to identify novel target genes of ER71. This analysis was done in collaboration with Prof. Tomas Pielers lab at the University of Göttingen.

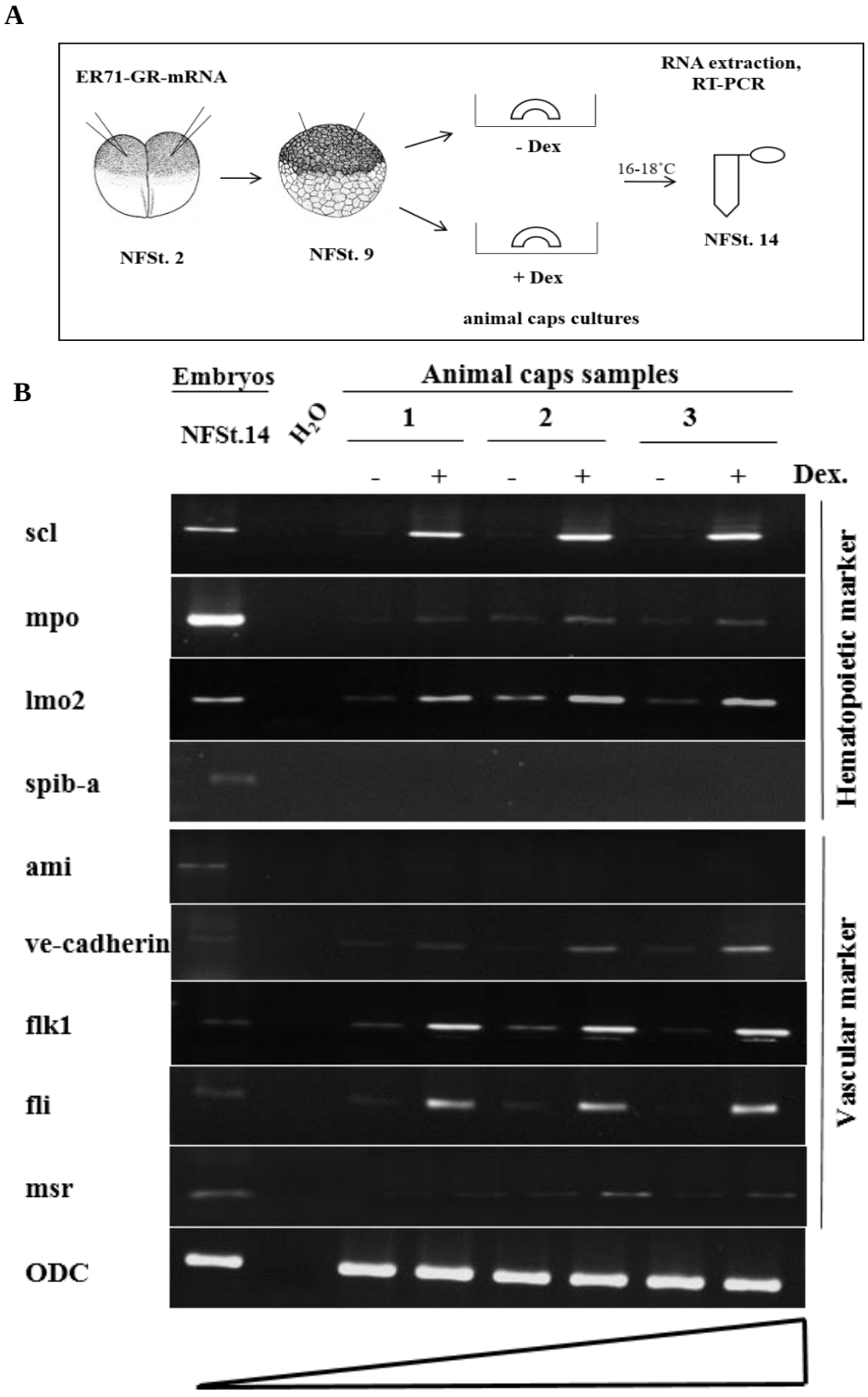

Figure 4.10 Induction of vascular and hematopoietic markers in ER71-GR overexpressing animal caps. (A) Experimental scheme, embryos were injected with 150 pg ER71-GR mRNA in both blastomeres at 2-cell stage. When the embryos reached NF St. 9, the animal caps were dissected, then incubated with and without Dex until they reached NF St. 14. They were collected for RNA extraction and RT-PCR was made using primer pairs to detect vascular and hematopoietic markers. (B) RT-PCR with total RNA extracted from animal caps at NF St. 14. RNA of whole embryos at NF St. 14 was used as a positive control and H_2O was used as a negative control. Ornithine decarboxylase (ODC-1) was used to demonstrate that comparable amounts of cDNA were used in each cDNA preparation.

4.9 The Conformation of newly ER71 target genes

Using deep sequencing technology, a large number of genes could be identified, that were upregulated in dexamethasone stimulated ER71-GR expressing animal caps compared to un-stimulated caps. To validate these results we choose six of these genes and performed semi-quantitative RT-PCR using cDNA prepared from an independent animal cap experiment. For three of these (*egfl7, exoc3l2* and *cplx2*) we could confirm that their expression was clearly upregulated in dexamethasone treated animal caps (Figure 4.11). For the other three genes (*spia1*, *camk2b* and *grip2*) we could not amplify specific PCR products, neither in untreated or in treated animal caps. At the moment we cannot say, whether this is due to technical problems or whether those genes are not expressed in the samples we analyzed. However, we could show that using deep sequencing technology, we could identify *cplx2*, *egfl7* and *exoc3l2* as potential novel target genes of ER71.

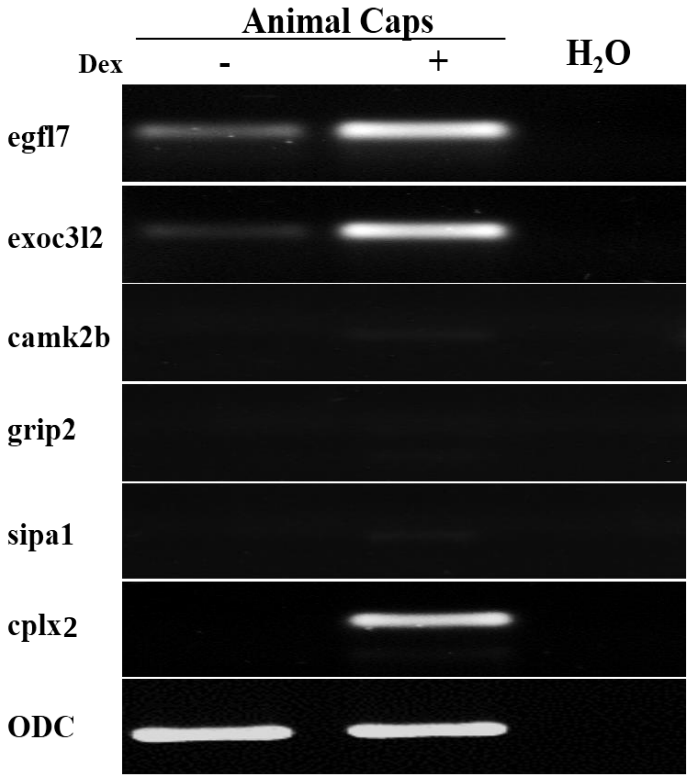

Figure 4.11 Verification of RNA-seq. data. Embryos were injected with 150 pg of ER71-GR mRNA in both blastomeres at 2-cell stage. When the embryos reached NF St. 9, the animal caps were dissected, then incubated with and without Dex until they reached NF St. 14. The total mRNA was extracted from animal caps for deep sequencing and RT-PCR. (egfl7) Epidermal Growth Factor like domain 7, (exoc3l2) Exocyst complex component 3-like 2, (camk2b) Calcium/calmodulin-dependent protein kinase type II beta chain, (grip2) Glutamate receptor-interacting protein 2, (sipa1) Signal-induced proliferation-associated protein 1, (cplx2) Complexin-2.

Table 4.2 The transcription factor genes examined from the RNA-seq. ER71 overexpression dataset

Gene ID	Gene description	Gene functions	The reads		Fold induction
			-Dex	+Dex	
Fli1	Friend leukemia virus integration 1	Transcription factor	1021	6100	6
Tal1	T-cell acute lymphocytic leukemia 1	Transcription factor	416	5191	12
Zeb2	Zinc finger E-box-binding homeobox 2-like	Homeodomain transcription factor	227	1829	8
Lmo2	LIM domain only 2 (rhombotin-like 1)	Transcription factor	378	1725	5
Traf4	TNF receptor-associated factor 4	Zinc finger, TRAF type	294	1778	6
Hhex	Hematopoietically expressed homeobox	Homeodomain transcription factor	367	1560	4
En2	Engrailed homeobox 2	Homeodomain transcription factor	11	786	69
Gfi1	Growth factor independent 1 transcription repressor	zinc finger, C2H2 type	38	769	20
Mef2d	Myocyte enhancer factor 2D	MADS-box transcription factor	410	2790	7
Tek	TEK tyrosine kinase, endothelial	Endothelial cell receptor	100	695	7
Hoxb3	Homeobox B3	Homeodomain transcription factor	63	428	7
Nkx3-1	NK3 homeobox 1	Homeodomain transcription factor	49	402	8
Nkx2-6	NK2 homeobox 6	Homeodomain transcription factor	57	233	4
Mnx1	Motor neuron and pancreas homeobox 1	Homeodomain transcription factor	13	682	53
Wt1	Wilms tumor 1	Zinc finger transcription factor	19	618	33
erf	Ets2 Repressor factor	Repressor factor	13	208	16
Ptf1a	Pancreas specific transcription factor, 1a	Transcription factor	23	149	7

Table 4.3 The migration, exo/endocytosis and adhesion genes examined from the RNA-seq. ER71 overexpression dataset

Gene ID	Gene description	Gene functions	The reads		Fold induction
			-Dex	+Dex	
Egfl7	EGF-like-domain7.	Vascular tubulogenesis in vivo.	127	2103	17
Smtn	SMTN (smoothelin)	Growth differentiation factor	1689	5842	3
Exoc3l2	Exocyst complex component 3-like 2	Required for proper VEGFR-2 signaling	227	3889	17
Emilin1	Elastin Microfibril Interfacer 1	Development large blood vessels	162	1448	9
Cplx2	Complexin-2	Synaptic vesicle exocytosis.	96	1814	19
Rin1	Ras and Rab interactor 1	Signal transduction / endocytosis	7	64	9
Rgl2	Ral guanine nucleotide dissociation stimulator-like 2	Guanine-nucleotide releasing factor	298	3021	10
Sipa1	Signal-induced proliferation-associated 1	Induced GTPase activating protein (GAP)	57	1223	21
Ap3s2	Adaptor-related protein complex 3, sigma 2 subunit	Endozytosis	39	334	9
Nrap	Nebulin-related-anchoring protein-like	Actin binding protein with lim domain	44	451	10
Pear1	Platelet endothelial aggregation receptor 1	Platelet receptor	60	349	6
Vegfb	Vascular endothelial growth factor B	Peptide growth factor	12	70	6
Apln	Apelin	Secreted peptide angiogenic	34	150	4
Dll4	Delta-like 4	Signal transduction/notch signaling pathway	35	302	9
Pecam1	Platelet/endothelial cell adhesion molecule 1	Immunoglobin and related proteins	7	72	10
Pkp3	Plakophilin 3	Neural adherens junction protein	2163	5621	3
Esam	Endothelial cell-specific adhesion molecule	Endothelial cell-specific adhesion molecule	28	164	6
Pcdh12	Protocadherin 12	Cadherin repeats	68	470	7

Table 4.4 The signal receptor genes examined from the RNA-seq. ER71 overexpression dataset

Gene ID	Gene description	Gene functions	The reads		Fold induction
			-Dex	+Dex	
Flt4	Fms-related tyrosine kinase 4	Vascular endothelial growth factor receptor flt-4	78	1131	15
Kdr	Vascular endothelial growth factor receptor kdr-like/ Flk-1	Vascular endothelial growth factor receptor 2	264	1096	4
vegfr-l	Vascular endothelial growth factor receptor 1	Endothelial cell mitogenesis and cell migration	24	137	6
ephb2	EPH receptor B2	Ephrin Receptor B2	759	5280	7
Plxnd1	plexin D1	Semaphorin receptor	458	2593	6
Plxnb3	plexin B3	Semaphorin receptor	190	1229	6
Grip2	glutamate receptor interacting protein 2	PDZ domain, Xgrip2.1|Xgrip2	67	1350	20
Nrp1	Neuropilin 1	Transmembrane receptor	48	299	6
Neto1	Neuropilin (NRP) and tolloid (TLL)-like 1 (neto1)	Brain specific	16	103	6
Tie1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	Angiogenesis	203	1936	10
Camk2b	Calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	serine/threonine kinase, cam2|camkb|camk2	201	4971	25

5. Discussion

ETS transcription factors are essential for development and regulation of endothelial cell behavior. Recent studies have revealed a central role for ETV2/ER71 during initial specification and differentiation of the endothelium (Meadows at el., 2011). It seem likely that ER71 activates the endothelial transcriptional program, which includes other ETS transcription factors, and that these factors assume regulation of endothelial gene expression when ER71 is downregulated. While the importance of ETS proteins for endothelial development is clear, the mechanism by which target gene specificity is achieved remains unclear. In this study we have used gene expression profiling, to identify a set of genes that are induced by ER71 during early development. Among of these genes are vascular and myeloid genes.

5.1 ER71-glucocorticoid receptor fusion protein whose activity can be regulated with dexamethasone treatment

Overexpression of ER71 during early embryogenesis causes severe gastrulation problems, which made it very difficult to analyze the effects of ER71 overexpression in whole embryos (Neuhaus et al., 2010). To overcome this problem, we constructed a dexamethasone-inducible ER71 protein where the entire ER71 coding region is fused with the ligand-binding domain of the glucocorticoid receptor. This construct allows the overexpression of ER71 in all the cells of the embryo. However, the fusion protein is retained in the cytosol, due to the binding of the GR-part to hsp90, thereby inhibiting transcriptional activity in the nucleus. The transcriptional activity of ER71 could then be regulated temporarily by adding dexamethasone to the culture medium. This releases the protein from hsp90 and enables its translocation into the nucleus (Gammill and Sive, 1997). The use of inducible constructs may assist in the search for target genes (Tada et al., 1997). In transfection experiment using Hela cells, we could show that the translocation of the ER71-GR protein into the nucleus was strictly dexamethasone dependent, showing that the construct worked as expected (Figure 4.2). Similar results were also obtained in animal caps that were injected with the ER71-GR fusion construct (Figure 4.7). However, there was some induction in untreated animal caps which might be due to the limited number of hsp90 molecules in the cells which were not enough to bind all ER71-GR protein.

5.2 The inducible ER71-GR construct can be used to analyze the ectopic induction of endothelial and myeloid markers genes in *Xenopus* embryos

To analyze whether the inducible ER71-GR construct can be used to analyze the expression of ER71 target genes during later development, we injected the construct into one blastomere of 2-cell stage embryos and induced the translocation of ER71-GR to the nucleus by adding dexamethasone to the medium either at NF St. 10 or at NF St. 20 (Figure 4.3 B, C). In embryos induced at NF St.10 the induction of hematopoietic marker *lmo2* could be detected already at NF St. 14, whereas the vascular marker gene *ami* was not detectable because it is not expressed at this stage endogenously and is also not induced by the construct at this stage, may be due to the expression pattern of *ami*, which were detected at later neurula stage (NF St. 16) (Inui and Asashima, 2006). In embryos treated with dexamethasone at NF St. 20 an ectopic induction of both marker genes, *lmo2* and *ami*, could be detected at NF St. 34. This result demonstrated that overexpression of ER71 could induces the vascular and the hematopoietic markers genes (Neuhaus et al., 2010). To extend the temporal inducibility of *ami* further we made a series of experiments where we added dexamethasone to the buffer of injected embryos at NF St. 12, 14, 18 and 20 and analyzed the induction of *ami* (Figure 4.4 B) and *mpo* (Figure 4.5) expression at NF St. 34. We could show that the extent of overexpression increased the later dexamethasone was added, showing that the number of hemangioblasts responsive to ER71 increased between NF St. 10 and NF St. 20. These results are in accordance with published data for other ets-family members and for studies performed in different model systems. The ETS family protein, ERG, has the ability to induce ectopic expression of endothelial markers in *Xenopus* embryos (Baltzinger et al., 1999; Meadows et al., 2009). This property is shared by the zebrafish ETV2 ortholog, which is able to activate expression of numerous endothelial genes in zebrafish embryos (Sumanas and Lin, 2006; Gomez et al., 2009; Wong et al., 2009).

Our studies show that forced expression of ER71-GR in *Xenopus* also activates transcription of endothelial and hematopoietic marker genes, including *ami*, *lmo2* and *mpo*. Associated with ectopic marker activation, overexpression of ER71-GR in the *Xenopus* embryo resulted in interference with normal vascular patterning. This is most clearly showed by the absence or altered structure, of intersegmental vessels or overgrowth of the vascular plexus in anterior ventral regions of the embryo. These observations suggest that levels of ER71 must be regulated within narrow limits for normal vascular development (Salanga, et al., 2010).

5.3 Identification of novel ER71 target genes

ER71 is important for the specification of endothelial and hematopoietic lineages. To understand how ER71 controls development of hematopoietic and endothelial lineages, it is essential to identify the direct targets of ER71 during early vascular development, which will lead to greater insight into vasculogenesis and hematopoiesis, and may help to identify therapeutic targets to treat vascular disorders or to promote or inhibit vessel growth (Lammerts van Bueren and Black, 2012). Several studies have sought to identify target gene using animal caps system (Gammill and Sive, 1997).

The formation of three germ layers is the most important event in early vertebrate development. Animal cap cells represent prospective ectodermal cells, but give rise to endodermal, mesodermal and neuro-ectodermal derivatives if treated with the appropriate factors (Borchers and Pieler, 2010).

Animal cap assays can be used to estimate the inducing activity of soluble factors at the histological and molecular level (Okabayashi and Asashima, 2003). The competence of animal caps cells are restricted to a specific germ layer at the onset of gastrulation (stage 9) (Domingo and Keller, 2000; Green and Smith., 1990; Kengaku and Okamoto, 1995; Lamb and Harland, 1995). Our observations are also consistent with previous studies of ER71 overexpression in animal caps, showing that forced expression of ER71 activates the transcription of a number of known vascular and hematopoietic marker genes as *scl*, *ve-cadherin*, *flk1*, *fli*, *msr*, *ami*, *lmo2* and *mpo* (Neuhaus et al., 2010, Salanga et al., 2010). However, these overexpression studies did not allow to determine which of the endothelial and hematopoietic genes are direct targets of ER71 regulation. To address this question we wanted to use the inducible ER71-GR construct in combination with cycloheximide, an inhibitor of translation (Kadener et al., 2007). Therefore, we injected ER71-GR sense-RNA into both cells of 2-cell stage embryos, let them develop until NF St. 9 and dissected region located at the animal pole, the so called animal cap, which are considered to show many characteristics of embryonic stem cells.

Those animal caps were treated with cycloheximide to prevent protein translation and subsequently dexamethasone was added to allow ER71-GR to enter the nucleus where the ER71 transcription factor could stimulate transcription of its target genes (Gammill and Sive, 1997). The RNA was used to control the induction of known target genes by RT-PCR. Despite numerous attempts we were not able to establish conditions leading to a significant induction of ER71 target genes. It is known that some genes are expressed in the presence of cycloheximide (Fukui and Asashima, 1994). Therefore, we omitted the treatment with cycloheximide. In ER71-GR overexpressing animal caps treated with dexamethasone we could reproducibly detect the

induction of known ER71 target genes (Figure 4.10 B) (Neuhaus et al., 2010). To identify novel ER71 target genes we applied RNA deep sequencing technology in a collaboration with Prof. Tomas Pielers group in Göttingen. Using deep sequencing we could identified numerous genes that were upregulated in dexamethasone stimulated animal caps overexpressing ER71-GR compared to not dexamethasone treated animal caps (Figure 4.11). This list contained a number of known ER71 target genes like *fli1, lmo2, scl* and *flt1*, confirming that the treatment of the animal caps with dexamethasone lead to the successful induction of ER71 target gene expression. We also identified a number of genes as ER71 targets, which are already known to play a role during vasculogenesis and hematopoiesis, like *hhex, kdrl, tie1, egfl7, vegf-b, exoc3l2, camk2b, grip2,* and *epo*.

For example, it has been shown that *Egfl7* is involved in vascular development in *Xenopus* embryos (Nagamine et al., 2005; Nichol and Stuhlmann, 2012) and loss function of Egfl7 in zebrafish embryos revealed that it regulates vascular tube formation (Parker et al., 2004, Gomez et al., 2012). Expression of Egfl7 in tumors promotes tumor progression by reducing the expression of endothelial molecules that mediate immune cell infiltration (Delfortrie et al., 2011). In mice Egfl7 physically interacts with endothelial notch and that it is capable of modulating notch signaling *in vivo* (Nichol et al, 2010). *Exoc3l2* is indeed important for vessel formation and/or function. Exoc3l2 function is required for VEGFR2 activation and directional migration of endothelial cells in response to gradients of VEGFA. Vascular expression of exoc3l2 was confirmed by RT-PCR analysis of different mouse tissues and immunofluorescence analyses of mouse brain sections (Barkefors at el., 2011). Ca^{2+}/calmodulin-dependent kinase II (CaMKII) phosphorylates serine residues in the auto-inhibitory domain for DNA binding of the transcription factor Ets1, leading to a negative regulation of GM-CSF transcription (Liu and Grundstrom, 2002). The human gene for complexin 2 (*grip2*) is expressed in the murine brain vessels and in the vessels of other organs, including the kidney, heart, skeletal muscle, and mesentery (Fouillade et al., 2013).

All these genes can now we added to the list of genes that are upregulated upon ER71 activity during vasculogenesis and hematopoiesis. Most importantly however, we could also identify ER71 target genes with interesting functions, which were not known to be involved in vasculogenesis so far. *Cplx2,* is neuron specific and codes for a protein involved in the exocytosis of synaptic vesicles (Raevskaya et al., 2005). *March2* is an E3 ubiquitin-protein ligase, also known as membrane associated ring finger (C3HC4) 2. In cell culture experiments it was shown that MARCH2 promotes endocytosis and lysosomal sorting of carvedilol-bound β (2)-adrenergic receptors (Han et al., 2012). The Ral guanine nucleotide dissociation stimulator-

like 2 (Rgl2) is a member of the RalGDS family that modulates expression of hypertrophic genes in cardiomyocytes. Increased expression and function of Rgl2 in cardiomyocytes of Rgl2 transgenic mice promotes activation of the PI3-kinase signaling cascade and protects from carciomyocyte death and pathologic cardiac fibrosis (Scotland et al., 2013). The DNA-binding transcription factor Smad-interacting protein-1 (Sip1) (also named Zfhx1b/ZEB2) plays essential roles in vertebrate embryogenesis. In *Xenopus*, XSip1 is essential at the gastrula stage for neural tissue formation (van Grunsven et al., 2007).

Since it was known that the detection of false positives in various expression analysis platforms can be a common problem, we selected a set of six potential new ER71 target genes and analyzed their expression with RT-PCR experiments on independent mRNA samples from animal caps, injected with ER71-GR mRNA and treated with and without dexamethasone. For three of the genes that we analyzed (*cplx2*, *exoc3l2* and *egfl7*) we could show that they are clearly upregulated upon dexamethasone treatment. The other three genes (*camk2b*, *grip2* and *sipa*) were not detectable at all, which could be due to technical problems. The analysis demonstrated that 50% of the potential ER71 target genes could be verified in an independent experiment, showing that the data from the deep sequencing analysis is very reliable. In summary, our analysis led to identification of large number of new ER71 target genes, which can now be placed in the regulatory network required for proper vasculogenesis.

6. Summary

Vasculogenesis and the formation of a functional vascular network are essential for embryonic development. Recently, the ETS domain transcription factor ETV2/ER71 has been identified as an essential regulator of vasculogenesis. Since it has been shown that overexpression of ER71 during early embryogenesis causes severe gastrulation problems, which made it very difficult to analyze the effects of ER71 overexpression in whole embryos, I constructed a dexamethasone-inducible ER71. This construct allowed the analysis of the effect of ER71 overexpression at later stages of *Xenopus* development without facing gastrulation problems and I could analyze the temporal accessibility of the promoter regions of two different ER71 target genes, *ami* and *lmo2*. With this analysis I could show that the number of hemangioblasts responsive to ER71 increased between NF St 10 and NF St 20.

The main objective of this thesis was the identification of novel ER71 target genes. I used the inducible ER71-GR construct to overexpress ER71-GR in animal caps, which were then treated with dexamethasone. I could reproducibly detect the induction of known ER71 target genes. To identify novel ER71 target genes I applied RNA deep sequencing technology in a collaboration with Prof. Tomas Pielers group in Göttingen. Using deep sequencing we could identified numerous genes that were upregulated in dexamethasone stimulated animal caps overexpressing ER71-GR compared to not dexamethasone treated animal caps. This list contained a number of known ER71 target genes like *fli1, lmo2, scl* and *flt1*, confirming that the treatment of the animal caps with dexamethasone lead to the successful induction of ER71 target gene expression. I also identified a number of genes as ER71 targets, which are already known to play a role during vasculogenesis and hematopoiesis, like *hhex, kdrl, tie1, egfl7, vegf-b, exoc3l2, camk2b, grip2,* and *epo*.

Most importantly however, we could also identify ER71 target genes with interesting functions, which were not known to be involved in vasculogenesis so far, including *march2 rgl2, zeb2, cplx2, exoc3l2* and *egfl7*. 50% of the potential ER71 target genes could be verified in an independent experiment, showing that the data from the deep sequencing analysis is very reliable. In summary, our analysis led to identification of large number of new ER71 target genes, which can now be placed in the regulatory network required for proper vasculogenesis.

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8. Theses

1. *Xenopus* ER71 is involved in vascular development.

2. The transcriptional factor ER71 interferes with the gastrulation in *xenopus* embryos.

3. The inducible ER71-GR construct can be used to analyze the ectopic induction of endothelial and myeloid markers genes (*ami, lmo2, mpo*) in *Xenopus* embryos.

4. The translocation of Myc-ER71-GR protein from the cytosol to the nucleus in Hela cells and animal caps is dependent on dexamethasone treatment.

5. Overexpression of ER71 induced the expression of vascular and hematopoietic marker genes in *Xenopus* embryos as well as animal caps.

6. The number of hemangioblasts responsive to ER71 signalling increased between NF St. 10 and NF St. 20.

7. ER71-GR induced the hematopoietic marker *lmo2* in animal caps at NF St. 14.

8. The endogenous expression of ER71 is up regulated in treated animal caps.

9. Using RNA deep sequencing technology, we could identify a new novel ER71 target genes.

10. Identification of a large number of new ER71 target genes, which can now be placed in the regulatory network required for proper vasculogenesis.

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Affidavit

Herewith I declare, that I prepared the PhD thesis “Identification of new target genes of the transcriptional regulator ER71” on my own and with no other sources and aids than quoted. I only have this application towards the commencement of examination procedures and no other promotion process at another university or college in or outside Germany.

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