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INDUCIBLE DOMINANT NEGATIVE *ErbB2* RAT SPERMATOGONIAL LINE FOR GENERATION OF TRANSGENIC RAT MODEL AND DISSECTING ERBB2 TYROSINE KINASE MEDIATED PATHWAYS

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Aim: **The ERBB2 receptors tyrosine kinase, also known as HER2/Neu, play an essential role in early organism development and modulation of cell behavior in varieties of tissue in an adult organism. Our *aim* was the generation of transgenic rat spermatogonial line capable of inducible expression of a dominant negative form of the ERBB2 protein *in vitro* and the transgenic rat as an animal model for dissection of the ERBB2 mediated signaling *in vitro* and *in vivo*. *Materials and Methods:* Donor derived rat spermatogonial stem cells that express green fluorescence protein and inducible ERT2CreERT2 recombinase were modified with Sleeping Beauty transposon-based vector that express truncated kinase deficient form of the ERBB2 receptor upon Cre mediated recombination. Clonally selected spermatogonial cell lines were extensively tested *in vitro*. Animals were generated via spermatogonia mediated transgenesis by transplantation of clonal cell line into testes of chemically sterilized recipients. Obtained progeny were tested for inducibility *in vivo* and served as donors of spermatogonia for downstream analysis. *Results:* We obtained animals and clonally derived spermatogonial stem cell lines that express an inducible dominant negative form of the ERBB2 protein. Isogenic nature of induced and uninduced cells allows most accurate morphological and molecular comparison of cells affected by the interruption of normal function of the ERBB2 receptor and cellular dynamics *in vitro* and *in vivo*. *Conclusions:* Clonally derived spermatogonial stem cell lines that express an inducible dominant negative form of *ErbB2* demonstrated an obvious difference in morphological appearance and growth kinetics of induced cells comparing to uninduced ones. Western blot analysis of induced and uninduced cells revealed significant differences in presence and phosphorylation state of several tested important proteins involved in the ERBB2 mediated signal transduction, such as S6 ribosomal protein; AKT (the serine/threonine kinase also known as protein kinase B); and three protein kinases that participate in the RAS-RAF-MEK-ERK signal transduction cascade PRAS40, MEK, ERK1/2.**

Key Words: **ERBB2, HER2/Neu, cancer, spermatogonial stem cell, clonal analyses, transgene expression, transgenic rat.**

The ERBB2 related transmembrane proteins comprise a family of protein tyrosine kinases (PTKs) that are essential signal transducers in many cell types in all vertebrates [1–8]. The family includes four proteins (ERBB1-4) and their isoforms that can form at least 26 different homo- and heterodimers as functionally active units [9]. Combined with eleven growth factors [9] as ligands initiating protein dimerization, these receptors can generate a large number of signaling events that regulate many aspects of cellular behavior including growth and apoptosis, proliferation and differentiation, motility and migration. Amplification, over-expression, or mutation of the ERBB2 family members and/or their ligands often associated with different forms of cancer, hence, making them important subjects for research [10–15].

ERBB2 protein, also known as HER2/Neu, is an outstanding member of the RTK family, since it has no known ligands but is the preferred heterodimerization partner for the rest of the family members [16]. This receptor is estimated to be overexpressed and/or amplified in 15–20% of aggressive breast cancers [17, 18]. Therefore, the biology of ERBB2 related metabolic processes is under wide-ranging investigation. At the same time, expression of this protein is detected in spermatogenic cells during their rapid proliferation up to primary spermatocyte stage [19].

Embryonic or perinatal lethality of *ErbB2* null mutants in model organisms [20–23] does not allow the traditional study of the gene function *in vivo*. Attempts were made to study the function of the protein *in vitro* and *in vivo* using models, with overexpression of intact or truncated forms of ERBB2 protein [24–31]. High level of natural expression of ERBB2 in spermatogenic cells in an adult organism provides an opportunity to investigate additional aspects of ERBB2 facilitated pathways under habitual conditions. Beneficially, spermatogenic cell culture can serve not only as a model to investigate biological processes *in vitro* but can be utilized for spermatogonia mediated transgenesis that has a potential to generate genetically engineered organism in a wide variety of animal species for research *in vivo* [32]. Availability of more appropriate animal models can provide not only constant source of stable cell lines but bring the study on the level of the whole organism. Currently developed mice models are not quite adequate for investigation of ERBB2 role in tumorgenicity since they utilize expression of the truncated transgene in excitable and therefor mitotically inactive tissues such as muscles [33–35] and nerves [36]. As terminally differentiated cells they cannot be maintained in pro-

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Abbreviations used: PTKs – protein tyrosine kinases; SG – spermatogonia growth.

liferating cell culture. Alternatively, and conveniently, spermatogonial stem cells can be maintained in the culture during many passages. We propose a model which allows investigation of ERBB2 biology in epithelial tissue with high natural rate of cell division and *ErbB2* expression. Utilised inducible competitive inhibition approach allows direct comparison of cell populations from the same animal, on the same passage, in the same culture and treatment conditions that are different only in the expression of the activated transgene. Moreover, rats closer to humans in respect to physiology and therefore this species is more translatable as a model animal and preferable as test subjects for preclinical studies [37].

Here we report a creation of genetically engineered rat spermatogonial cell lines and animals that are capable of inducible DNA recombination followed by an expressing of a transgene that in our case is dominant negative form of ERBB2 protein. Chosen inducible recombination system provides several advantages over simple viral or plasmid modifications: firstly, in uninduced state transgene is not expressed and thus does not have any influence on organism development or cell metabolism; secondly, induction of the transgene is controllable an can be performed *in vivo* and *in vitro* at specific stages in time-dependent preplanned manner; thirdly, isogenic nature of created inducible system allows most accurate comparison of cells in induced *vs* uninduced state thus eliminating any biases related to the cell origin, isolation/cultivation conditions, passage number etc. The rat was produced via spermatogonia mediated transgenesis. Spermatogonial stem cells derived from double transgenic GCS-EGFP/DAZL-ERT2CreERT2 rat [32] were transformed with Sleeping Beauty transposon-based vector harboring a dominant negative form of *ErbB2* coding sequence. The protein can be expressed upon 4OH-tamoxifen inducible nuclear translocation of recombinant ERCreER followed by recombinase-mediated removal of floxed neomycin selectable marker sequence located between modified *DAZL* gene promoter and the sequence of dominant negative *ErbB2*.

MATERIALS AND METHODS

Animal care and use. Protocols for the use of rats in this study were approved by the Institutional Animal Care and Use Committee at UT-Southwestern Medical Center in Dallas, as certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. Rats used for this study were housed in individually ventilated, Lab Products 2100 cages in a dedicated room with atmosphere controls set to 22 °C, 45–50% humidity during a 12-hour light/dark cycle (i.e. light cycle = 6:00 a.m.–6:00 p.m., Central Standard Time adjusted for daylight savings time). Rats were fed Harlan Teklad Irradiated 7912, LM-485 Mouse/Rat Diet, 5% fat Diet and a continuous supply of reverse osmosis water.

Isolating undifferentiated type A spermatogonia. Seminiferous tubules were isolated from testes of 23–24 day old homozygous SD-Tg (ROSA-EGFP), or WT Sprague Dawley rats (Harlan, Inc.). Rats of the SD-Tg(ROSA-EGFP) line were produced by pronuclear injection and are referred to as GCS-EGFP rats because they exhibit germ cell-specific expression of enhanced green fluorescent protein (EGFP) [38]. The tubules were enzymatically and mechanically dissociated into a cellular suspension to generate cultures of testis cells in serum-containing medium [39]. The testis cell cultures were then used to isolate enriched populations of laminin-binding spermatogonia following previously established methods, which detail how to first remove > 95% of somatic testis cells from the germ cell population by negative selection on plastic and collagen, before positive selection for the spermatogonial stem cells based on their ability to bind to laminin [39, 40]. By this procedure, the freshly isolated laminin-binding germ cell population contains > 90% undifferentiated, type A spermatogonia (PLZF+, DAZL+) in the single (~88%) or paired (~12%) cell state. Note, fractions of laminin-binding spermatogonia isolated by this procedure contain ~4% somatic cells, and ~5% differentiating spermatogonia plus spermatocytes. This procedure was used to isolate a parental transgenic rat spermatogonial line, GCS-EGFP/DAZL-ERT2CreERT2, which stably expresses an inducible form of CRE recombinase (iCRE) selectively in the germline of tgGCS-EGFP rats [38]. After selection on gelatin freshly isolated laminin-binding germ cell were harvested and then plated on irradiated DR4 Mouse Embryonic Fibroblasts (Bio Pioneer Inc.) in duplicate wells (~1.9 cm^2) to initiate the subculture of spermatogonial lines [40, 41]. Alternatively, cells from the cryopreserved population of the corresponding cell line were plated directly on DR4 Mouse Embryonic Fibroblasts [42, 43]. The cells were passed every 11–12 days onto a fresh monolayer of MEFs at $(1–3) \cdot 10^4$ cells/cm^2. The cells were fed every two days with fresh spermatogonia growth (SG) media [39].

DNA constructs. To generate stably modified spermatogonial cell lines transposon-based vectors *pSBLox-Neo-Lox-iDN-ErbB2* were constructed as follows: backbone of Sleeping Beauty transposable element [43] was used to clone-in 2138 bp of DAZL promoter sequence (amplified by PCR from germ cell cDNA) to construct vector *pSB-DAZL-MCS-CAG-LoxNeoLox-GFP* cassette was excised from *pCALNL-GFP* vector (Addgene plasmid 13770) and cloned into multiple cloning site of *pSB-DAZL-MCS* thus resulting in *pSBLox-Neo-Lox-iEGFP. DNErbB2* coding sequence was inserted in place of EGFP sequence by conventional cut-and-paste cloning method. The integrity of obtained vectors was confirmed by Sanger DNA sequencing. Additionally, we used *pM3A* vector [43]. expressing transposase. For positive selection of transformed spermatogenic cells, *pNeoΔTK* vector was prepared from *pNeoTK* (Invitrogen) by deletion of TK cassette.

Production of transgenic rat spermatogonial lines. Spermatogonia were mixed with a cocktail

of plasmid DNA (*pNeoΔTK*, *pM3A*, *pSBLox-Neo-Lox-iDN-ErbB2*) at a concentration of 1 μg DNA/10^5 cells and electroporated by the Neon® Transfection System (Invitrogen) according to the manufacturer's recommendation. Transfected cells were selected with SG media supplemented with 60 mg/ml of G418. G418 selection started at day 3 post transfection and continued for additional 8 days. Following G418 selection cells were harvested and divided into two parts: one part was plated at $15 \cdot 10^3$ cells/well on 6-well plate for colony pick-up; the second part of the harvested selected cells was cryopreserved as a backup. Cells plated at low density were cultivated for two weeks to allow the formation of colonies suitable for manipulation under the microscope with low magnification. Distinctive colonies were manually picked and individually transferred to a separate well on a 96 well plate with MEF feeder cells for future expansion and analysis. Next, cells from individual colonies were passed into two replicated 96 well plates. Two days after plating one set of replicate plates was supplemented with SG media containing 300 nm of 4-HO-tamoxifen for induction of Cre mediated recombination and thus activation of the transgene expression. After 8 days post induction with 4-HO-tamoxifen cells were fixed with 4% paraformaldehyde in phosphate buffer (0.1 M sodium phosphate, pH 7.2). Cells transfected with *pSBLox-Neo-Lox-iDN-ErbB2* were immunohistochemically stained with anti-V5 antibody to detect *iDN-ErbB2* transgene expression. Colonies with the highest number of V5+ clonal units were selected for clonal expansion and transgenic cell line establishment.

Production of germ cell-specific inducible dominant negative ErbB2 transgenic rat and cell lines. WT Sprague Dawley male rats (Harlan, Inc.) at 12 days of age were treated with a single dose of busulfan (12 mg/kg) in order to deplete the endogenous population of spermatogonia and enhance engraftment of T138 spermatogonia line transformed with *SB-DAZL-ERT2CreERT2*. At 24 days of age, animals were transplanted with $350 \cdot 10^3$ of modified cells per testis according to the established protocol. Upon reaching reproductive maturity animals with transplanted spermatogonia became recipient-producers capable of generating genetically modified spermatozoa and were mated to WT Sprague Dawley female rats (Harlan, Inc.). The obtained transgenic male offspring became donors of the testicular tissue for the establishment of cell lines for followed up experiments. Isolation of undifferentiated type A spermatogonia from the testicular tissue was conducted as described above.

Immunocytochemistry. Spermatogonial cultures were fixed for 10 min with 0.4 ml/well of 4% paraformaldehyde in phosphate buffer (0.1 M sodium phosphate, pH 7.2). Fixed cells were rinsed three times with PBS (0.8 ml/well). The cells were then permeabilized and nonspecific protein-binding sites were blocked by incubating the cells in PBS containing 0.1% (vol/vol) Triton X-100 and 1% wt/vol blocking reagent (Roche Applied Biosciences) for 1.5 h at 22–24 °C. The blocking reagent was removed, and the cells were incubated for 16 h at 22–24 °C in primary antibodies (0.4 ml/well). The anti-deleted in azoospermia-like (Dazl)-3 IgG were diluted to 250 ng/ml in blocking reagent, the mouse anti-human ZBTB16 IgG (Calbiochem, Inc.) were diluted to 1 μg/ml in blocking reagent the Mouse Anti-V5 Monoclonal Antibody (Invitrogen) were diluted 1:5000 in blocking reagent. After incubation in primary antibodies, the cells were washed three times for 5 min with PBS or TBST (0.8 ml/well) to remove unbound IgG. The cells were then incubated for 40 min at 22–24 °C in the solution of conjugated, secondary antibody (0.4 ml/well) diluted to 1 μg/ml in PBS containing Hoechst 33342. After incubation with secondary antibodies, the cells were rinsed three times for 5 min with PBS or TBST (0.8 ml/well) to remove unbound IgG and dye before viewing in fresh PBS (0.8 ml/well) using an inverted Olympus IX70 microscope.

Western blotting. Cell protein extracts were prepared by lysing cell pellets in the 1.5 ml tubes with 100 μl/ $100 \cdot 10^3$ cells of ice-cold lysis buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 protease inhibitor tablet/12.5 ml). The lysates were incubated on ice for 15–20 min and then centrifuged at 15,800 × *g* for 10 min at 4 °C in a microcentrifuge (Model# 5042, Eppendorf, Inc.), and the resultant supernatants were then stored at –80 °C. Frozen supernatant solutions were thawed on ice and then further clarified by centrifugation at 230,000 × *g* (tla-100.3 rotor, TL1000 ultracentrifuge, Beckman, Inc.) for 15 min at 4 °C. Protein samples were supplemented with sample loading buffer (Fermentas) and reducing agent (Roche). Prepared samples were fractionated (≈ 10 μg protein/lane) after loading into 15 well, Nupage 4–20% Bis-tris gels using Tris-Glycine running buffer with 0.1% SDS. Gels were transferred in the Invitrogen Xcell II Blot module (semi-wet apparatus) onto BA85 Nitrocellulose, 30 V for 1 h in Tris-Glycine transfer buffer containing 10% methanol. After transfer, nonspecific, protein binding sites were blocked by incubating membrane for 2 h at room temperature in blocking buffer: TBST 0.1% (Tris-Buffered Saline with 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 3,5% BSA. After blocking, blots were incubated with primary antibodies diluted in blocking buffer overnight at 4 °C. The mouse anti-Alpha-Tubullin (MU121-UC; BioGenex) was diluted 1:1000. The Phospho-HER3/ErbB3 (Tyr 1289) (21 D3) Rabbit mAb (# 4791; Cell Signaling) was diluted 1/500, ErbB-3 rabbit polyclonal abntybody (sc-285, Santa Cruz Biotechnology, Inc.) were diluted 1:500; Phospho-HER2/ErbB2 (Tyr 877) rabbit polyclonal Antibody # 2241; Neu (C-18) rabbit polyclonal antibody (sc-284, Santa Cruz Biotechnology, Inc.) were diluted 1:500; Phospho-Akt (Ser473) rabbit polyclonal Antibody (# 9271; Cell Signaling) were diluted 1:1000, Phospho-Akt (Thr 308) (C31 E5E) Rabbit mAb (# 2965 Cell Signaling) were diluted 1:500; Akt rabbit polyclonal Antibody (# 9272 Cell Signaling) were diluted 1:1000; Phospho-PRAS40 (Thr 246) (C77D7)

Rabbit mAb (# 2997 Cell Signaling) were diluted 1:500; PRAS40 (D23C7) XP® Rabbit mAb (# 2691 Cell Signaling) were diluted 1:500; Phospho-MEK 1/2 (Ser 217/221) rabbit polyclonal Antibody (# 9121 Cell Signaling) were diluted 1:500; MEK 1/2 rabbit polyclonal Antibody (# 9122 Cell Signaling) were diluted 1:500; Phospho-p44/42 MAPK (Erk1/2) (Thr 202/Tyr 204) rabbit polyclonal Antibody (# 9101 Cell Signaling) were diluted 1:500; p 44/42 MAPK (Erk1/2) rabbit polyclonal Antibody (# 9102 Cell Signaling); Phospho-S6 Ribosomal Protein (Ser 235/236) (91B2) Rabbit mAb (# 4857 Cell Signaling) were diluted 1:500; S6 Ribosomal Protein (5G10) Rabbit mAb # 2217 Cell Signaling) were diluted 1:500. Blots probed with the mouse primary antibodies were rinsed 3 × 5 min with TBST 0.1% at 22–24 °C. The blot probed with the rabbit primary antibody was rinsed 3 × 5 min with TBST 0.5% at 22–24 °C. After washing, blots were incubated in either IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211) and/or IRDye 680 RD Goat anti-Mouse IgG (LI-COR P/N 926-68070) secondary antibodies (1:50000 dilution) for 45 min at 22–24 °C, then rinsed again as above using TBST 0.1%, and finally detected with ODYSSEY® CLx infrared imaging system. Quantifications of protein band's signal intensity were performed with Image Studio™ Lite software designed specifically for Western blot quantification. The software provides accurate densitometry without altering raw data generated by ODYSSEY® CLx infrared imaging system.

Statistical analysis. The graph and the used standard error of the mean (SEM) were prepared using Microsoft Excel.

RESULTS

Production of inducible dominant negative ErbB2 transgenic rat spermatogonial lines. Spermatogonia stem cells were isolated from the cross between DAZL-ERT2CreERT2 and GCS-EGFP transgenic rats [32, 38]. Germ cell from this animal is capable of simultaneous expression of two recombinant proteins: 1) ERT2CreERT2 fusion protein inducible by 4-OH-tamoxifen and 2) EGFP. Green fluorescent protein enables traceability of the cells while inducible Cre recombinase delivers controlled recombination of transient or stably inserted DNA constructs containing Lox sites.

Isolated spermatogonia were mixed with a blend of a vector containing Sleeping Beauty transposase and *pSBLox-Neo-Lox-iDN-ErbB2* Sleeping Beauty transposon-based vector (Fig. 1, *a*). Such combination serves as an effective tool for introducing exogenous DNA situated between the recombination sites. After the electroporation cells were selected with the media containing G418. Since the vector had a neomycin selectable cassette, only modified cells were expected to survive. Colonies generated by survived cell were manually transferred to three 96-well plates for expansion and further analysis.

Clonal analysis and selection of spermatogonial lines expressing dominant negative ERBB2. The advantage of a spermatogonia mediated transgenesis is the ability to analyze the functionality of desired modification prior to generation of the actual animal. All of the 288 clonally selected colonies were split to replica plates. One set of the plates was used for the clonal expansion of spermatogonia while another set of the cell was analyzed for an intended functional performance of the modifying construct. One set clonally expanded culture intended for the analysis was treated with 4-OH-tamoxifen during one feeding at doses established previously [32] to induce expression of *iDN-ErbB2* transgene tagged with V5 tail. The clones were fixed, and colonies were probed with the antibodies against V5 tag. Fig. 2 demonstrates the results of the *in vitro* transgene induction and expression analysis of two clones that represent different efficiency of inductiveness/expression of *iDN-ErbB2* construct. Germ cell specific expression EGFP ensures the spermatogonial lineage of analyzed colonies, while the presence of V5 tag (stained red) indicates presence of the DN-ERBB2 protein. Indeed, such staining was localized to the cellular membrane where the modified receptor was anticipated to be found. While the clone # 2 demonstrated high degree of inducible *iDN-ErbB2* expression, clone # 31 although was found to be positive, had a suboptimal number of induced colonies.

Due to the random transposition and potential post-integration positional silencing of the constructs, not all the clones were expressing the transgene at the same level. Indeed, despite positive neomycin selection more than half of the selected colonies upon induction with 4-OH-tamoxifen did not express the protein at the level detectable by immunocytochemistry method. The rest of the clones demonstrated a variable degree of recombination efficiency and expressed DN-ERBB2 protein at different levels. We selected three clones with the highest inducible expression of the transgene for further expansion, analysis and transplantation. Clone # 2 was used to produce a triple transgenic rat: *GCS-EGFP/ERT2CreERT2/iDN-ErbB2*.

Production of inducible dominant negative ErbB2 transgenic rat. We utilized a spermatogonia mediated transgenesis to produce a model rat that would be able to express a dominant negative form of ERBB2 protein in germ cells *in vivo* and *in vitro* upon 4-hydroxytamoxifen stimulated induction of recombination event. Clonal line # 2 of modified spermatogonia was expanded and transplanted into the testes of the recipient young rats (Fig. 1, *b*). By the virtue of stem cell transplantation, we generated chimeric animals that became producers of modified spermatozoa and founders of transgenic animal lines. After maturation, chimeric males were mated with WT Sprauge Dawley female rats and transgenic progeny were obtained. Full characterization of the animal line is beyond the scope of this publication. Nevertheless, produced transgenic rats were successfully tested for the inducibility of iERBB2 *in vivo* (Fig. 3). These animals served as donors of modified

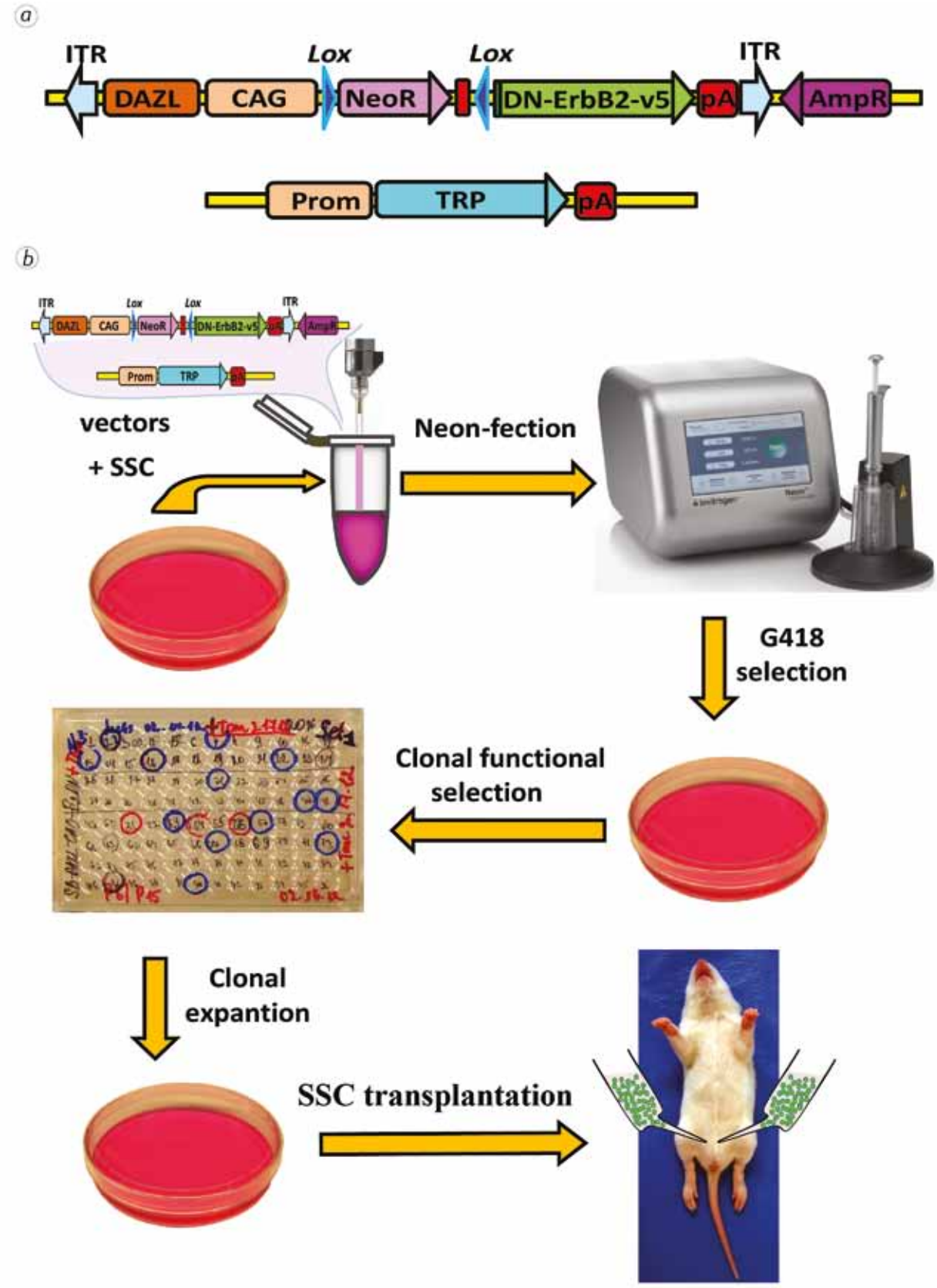

Fig. 1. Development of transgenic spermatogonial cell lines and an animal. (*a*) Vectors used for Sleeping Beauty mediated introduction of *iDN-ErbB2* for spermatogonia stem cell modification. (*b*) Technological steps in preparation of chymeric founder of transgenic animal line

cells for further investigations *in vitro.* Additionally this model animal can be used for the investigation of ERBB2 functional biology at organismal and organ level *in vivo*.

***In vitro* growth dynamics of triple transgenic *GCS-GFP/ERT2CreERT2/iDN-ErbB2* spermatogonial cell line.** Using obtained transgenic animals individual cell lines were initiated. Despite the indi-

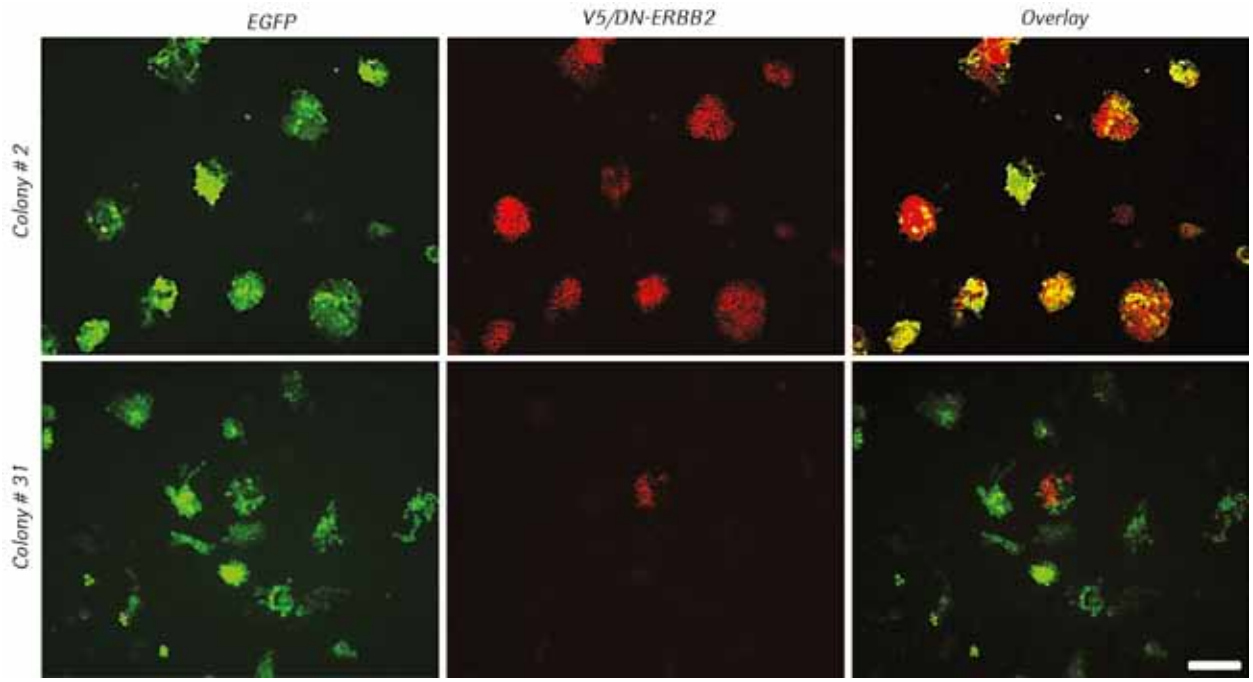

Fig. 2. Functional *in vitro* analysis of modified spermatogonial clones. Green indicates expression of EGFP. Red indicates presence of V5 tag thus iDN-ERBB2 protein localised to the cell membrane. Scale bar 100 μm

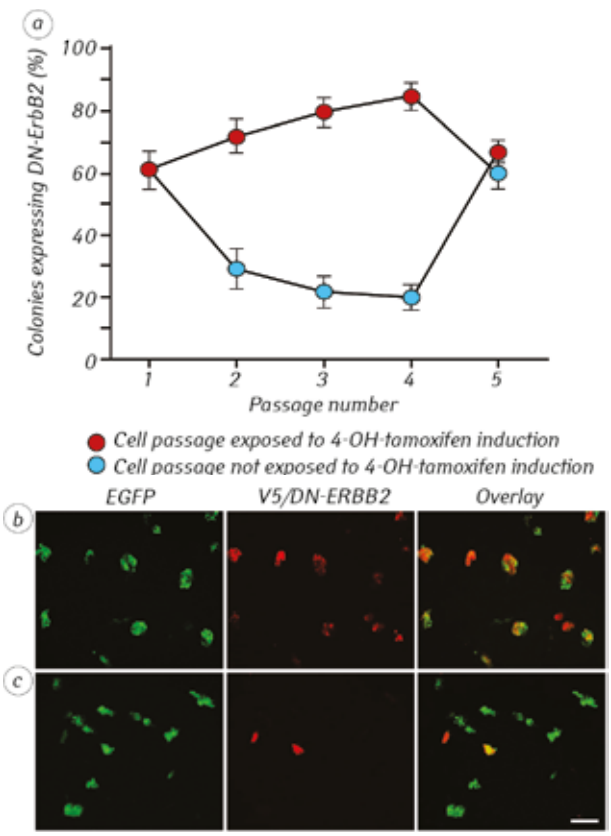

Fig. 3. Cell culture dynamics of the transgenic spermatogonia from clone # 2. (*a*) Fraction of the cell colonies expressing iDN-ERBB2 depending on number of 4-OH-tamoxifen inductions (± SEM, triplicate wells). (*b*) Representative appearance of the colonies (passage four) expressing iDN-ERBB2 in culture induced after each passage. (*c*) Representative appearance of the colonies (passage four) expressing iDN-ERBB2 in culture induced once at the beginning of the experiment. Scale bar 100 μm

vidual origin of the initiated cultures we estimated that only 60–65% of the cells were undergoing inducible recombination after any given exposure to the medium containing 4-OH-tamoxifen. Recombination efficiency of the loxed DNA fragment most likely depends on the accessibility of the chromosomal locus that is constantly changing depending on the cell cycle stage of the individual cell. The cellular exposure time to 4-OH-tamoxifen between passages was limited to one initial feeding that was approximately 48 h immediately followed by the passage. At the passage time, spermatogonia are intensively dispersed to a single cell suspension containing at most a few clusters with two or more cells. Thus, induction of the cell culture immediately after the passage provides the highest accessibility of the inducer to each individual cell. Despite such approach, we were not able to achieve 100% efficiency of recombination during any observed passage. Repeated exposure to 4-OH-tamoxifen at the beginning of each passage continuously increased the number of cells that underwent recombination. While maintaining a culture of the selected clones two observations were made: 1) a portion of the culture with induced expression of iDN-ERBB2 produced fewer spermatogonia between passages compare to the uninduced cells from the same clone cultured in parallel with approximately two-fold difference in the cell yield; 2) a fraction of induced cell after a single exposure to 4-OH-tamoxifen decreased rapidly with each passage while the number of uninduced cells increased. No leaky expression of iDN-ERBB2 in unindicted cells was observed.

An additional advantage of the male germ stem cell-mediated transgenesis is that a selected modified spermatogonia line itself can be utilized directly for further study. Indeed, the rat spermatogonia cell lines demonstrate chromosomal stability and resistance to differentiation. Such cell culture alone can be used to study protein function at a molecular level.

Using a temporal overexpression of truncated form of ERBB2 we achieved partial interruption of natural endogenous signal transduction. For a more detailed comparative analysis of induced and non-induced cells, an experiment was conducted that used a spermatogonial clonal culture after induction with 4-OH-tamoxifen during one feeding. In the subsequent passages, the culture was split into two parts — one part was induced with 4-OH-tamoxifen repeatedly after each passage. The second part was passaged without additional induction. Each of these parts was further divided into two subcultures: one of which was fixed after 10 days of cultivation for the immunohistochemical staining and counting the number of colonies expressing iDN-ERBB2. The second part of the subculture was further cultivated to increase the cells number until the next passage. This approach provided an accurate calculation of the fraction of positive colonies that directly reflect a number of cells proliferated in the previous passage. Since, during many passages, colonies maintained typical spermatogonia stem cell appearance, undifferentiating behavior and GFP marker as an indicator of spermatogenic origin we believe that "stemness" of tested cells was not interrupted. After ten days in culture, spermatogonial colonies had the appearance of the cell formations with distinct borders that are not touching and merging with each other. The percentage of colonies expressing the iDN-ERBB2 was counted after each passage. Observations were carried out during five consecutive passages. The experiment was conducted three times. It was established that after a single 48 h treatment of the cell lines with 4-OH-tamoxifen up to 60% of the colonies were expressing DN-ERBB2. Graph (Fig. 3, *a*) demonstrates the dynamics of cell cultures that express iDN-ERBB2 as a function of a different number of inductions after the passage. During the following passages, the percentage of colonies expressing transgene without additional stimulation was gradually decreasing while the other part of the culture subjected to 4-OH-tamoxifen treatment after each passage demonstrated a reciprocal tendency — percent of DN-ERBB2/V5 positive colonies increased after every passage (Fig. 3, *b* and *c*). At passage # 5 the parts of the cell culture with initial single 4-OH-tamoxifen exposure was treated once again and the part that received treatments every passage during four passages did not receive usual induction with media containing 4-OH-tamoxifen. The corresponding cultures demonstrated anticipated reciprocal response: without additional stimulation, percentage of developed colonies with iDN-ERBB2 expression decreased by more than 20%, while an additional treatment of the previously unstimulated culture resulted in an increase of V5 positive colonies by approximately 40%. The experiment demonstrated that a presence of colonies expressing iDN-ERBB2 requires repeated stimulation with 4-OH-tamoxifen for additional recombination. Without such stimulation number of iDN-ERBB2/V5 positive colonies decreasing with each consecutive passage. Interruption of the proper function of endogenous ERBB2 receptor clearly leads to impairing of the cell proliferation activity hence the unaffected cells with normal signal transduction by endogenous ERBB2 forming dimers during several passages were able to overgrow their counterparts affected by the transgene expression.

Apparently, an interruption of the normal function of ERBB2 receptor decreases the cell division rate and thus the proliferation of spermatogonia. A fraction of unmodified cells present in the culture was able to overgrow cells affected by iDN-ERBB2. The result of the final passage confirmed our preliminary observations: the lack of additional 4-OH-tamoxifen stimulation reduces the number of colonies in which the recombinant protein is expressed. Additional confirmation of such conclusions is the morphological appearance of the colonies without and with an expression of the recombinant protein. Visually, the unstimulated and therefore unaffected "normal" colonies had a higher number of individual spermatogonial stem cells per colony, and looked 2–3 or more times larger than induced counterparts of the same clonal origin, passage number and culturing conditions, with an expression of the truncated receptor (Fig. 4).

Comparative western blot analysis of GCS-EGFP/ERT2CreERT2/iDN-ErbB2 spermatogonial line in uninduced vs induced state. The dynamical behavior of cultures that differ in the presence or absence of iDN-ERBB2 indicates the relationship between this receptor and the processes associated at least with the regulation of the cell cycle and the mitotic activity of the spermatogonia. In order to test the applicability of the generated transgenic cell line/animal, we conducted western blot analysis. We analyzed the presence and functional state of some of the kinases that are believed to be involved in the metabolic pathways activated by the ERBB proteins in response to cell stimulation by the corresponding ligands: EGF (epidermal growth factor), TGF (transforming growth factor), AREG (amphiregulin), HBEGF (heparin-binding EGF-like growth factor), BTC (betacellulin), NRG1α1 (neuregulin 1α1) and NRG1β1 (neuregulin). Protein extracts from unactivated cells in standard culture conditions without any ligand served as a control for both total and phosphorylated proteins (Fig. 5).

For the testing purposes, we selected five proteins: S6 ribosomal protein; AKT (the serine/threonine kinase also known as protein kinase B); and three protein kinases that participate in the RAS-RAF-MEK-ERK signal transduction cascade PRAS40, MEK, ERK1/2. For each kinase, we analyzed the amount of total protein and the protein in the phosphorylated state in the cultures with the endogenous ERBB2 protein and culture affected by the expression of iDN-ERBB2. On all the blots V5 tag indicates the presence of iDN-ERBB2. It would be logical to expect that the expression of iDN-ERBB2 without a phosphorylation domain would have an effect on related proteins and/or the proportion of these proteins in the phosphorylated state. Indeed, even in the control culture without any additional stimulation with the selected ligands expres-

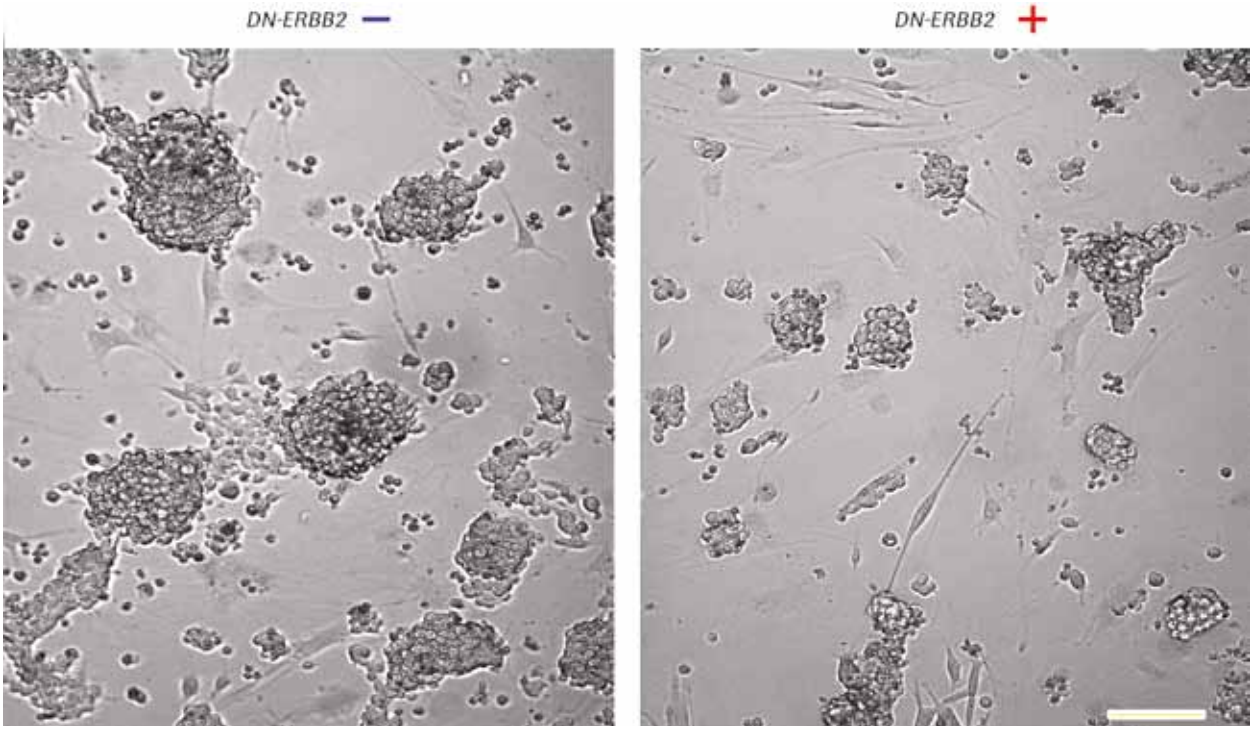

Fig. 4. Representative morphological apearence of spermatogonia without and with expression of DN-ERBB2. Scale bar 100 μm

sion of iDN-ERBB2 protein dramatically increased amount of S6 and MEK proteins in the phosphorylated state. While the amount of total S6 protein was insignificantly higher comparing to the control conditions, the amount of MEK total protein was always lower. In cells expressing iDN-ERBB2 amount of AKT protein decreased almost in half, however, despite such a decrease in culture stimulated with NRG1β1 quantity of AKP in the phosphorylated state was more than two times higher. Presence of iDN-ERBB2 marked an insignificant increase in total PRAS40 kinase but only in cultures stimulated with, HBEGF, BTC, NRG1α1, and NRG1β1 level of phosphorylated PRAS40 was notably higher. Interesting results were obtained for the final executioner or MAPK-ERK pathway ERK1/2. As one would expect interruptive interference with RTKS should decrease the activity of this protein and that is what can be observed in our experiment. Despite a slight increase in the amount of total protein the quantity of phosphorylated ERK1/2 in control conditions and in presence of any of the tested ligands is always lower in cells affected by the expression of iDN-ERBB2.

DISCUSSION

ERBB2 receptors tyrosine kinases is an important receptor that is directly involved in the regulation of cell cycle and many malignant tumors that exhibit an abnormal function of this protein. In the current study, we demonstrated an effective generation of the transgenic rat spermatogonial cell lines and animals with inducible expression of a dominant negative form of ERBB2 via modification of spermatogonial cells followed by transplantation into the recipient testes. The expression of the transgene was germ cell specific. The introduced recombinant protein is lacking phosphorylation domain and has V5 tag for identification purpose. The absence of phosphorylation activity of the protein impaired normal cell cycle and proliferation activity of spermatogonia *in vitro*. The doubling rate of the cells with 4-OH-tamoxifen stimulated activation of iDN-ERBB2 expression was more than twice as low as the doubling rate of cells with expression of endogenous ERBB2. The designed transgene successfully performed the intended function, namely interruption of the normal function of ERBB2. Assuming that iDN-ErbB2 entering into homo- or heterocomplexes with other partners in the family contributes to a decrease in mitotic activity in comparison with normal protein. The amplification, overexpression or constitutive activity of ERBB2 hyperstimulates the mitotic activity of cells observed in malignant neoplasms associated with the ERBB2 function. Created experimental conditions mimicked the situation in which the normal expression of ERBB2 behaves like overexpression comparing to cells expressing iDN-ERBB2. Cells with the normal activity of ERBB2 overgrow cells with active iDN-ERBB2. This observation raises the question of the effectiveness of the use of medicines aimed at obstructing the phosphorylation activity of ERBB2. It is self-evident that such drugs will only be effective at a constant stable concentration in the blood. In the event of a decrease in concentration or discontinuation of treatment, transformed or mutated cells can restore ERBB2 stimulated mitotic activity and quickly overgrow neighboring cells. Obviously, under such circumstances, the search for alternative ERBB2-kinase and kinase-associated targets and methods is an open question [44–48].

Western blot analysis of spermatogonia with and without the presence of iDN-ERBB2 demon-

strated a possibility for detailed pathway dissection of ERBB2 related signaling. Comprehensive analysis of the pathways and construction of metabolic map and molecular interactions would require a significant volume of additional experiments and thus is beyond the scope of this publication. Comparative microarray analysis of spermatogonia without and with the expression of iDN-ERBB2 would provide additional information on ERBB mediated cell signaling at the transcriptional level. Such information may lead to the development of conceptually new treatment strategies potentially targeting mechanisms of gene regulation rather than inhibiting gene products at the protein level.

We confirmed robust inducibility of the transgene expression *in vivo* (Fig. 6). Since iDN-ERBB2 can be effectively induced both *in vitro* and *in vivo*

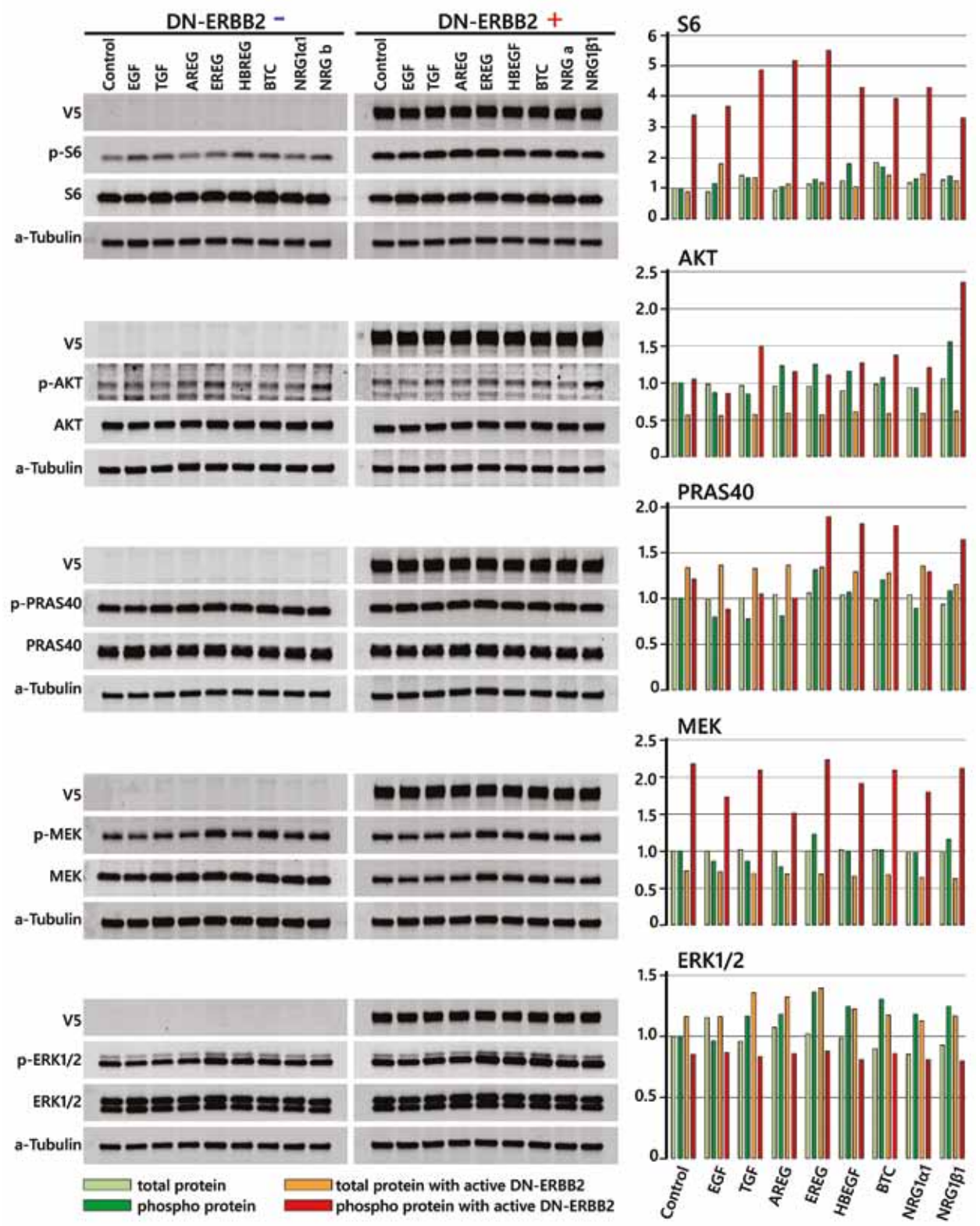

Fig. 5. Western blot analysis of selected spermatogonial proteins from uninduced cell culture (left panel) and cell cultures induced 4-OH-tamoxifen to express iDN-ERBB2 *in vitro*

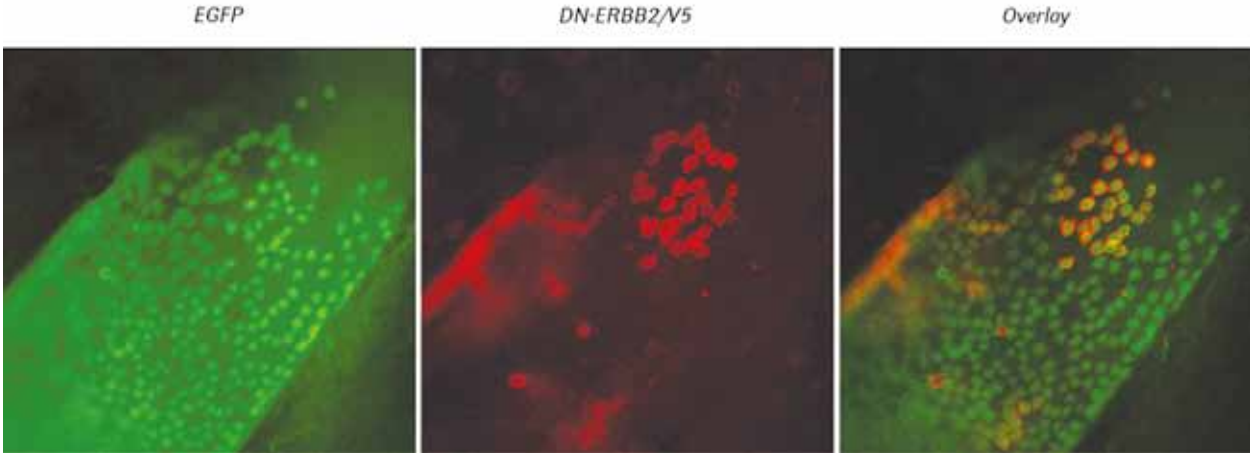

Fig. 6. Induction of iDN-*ErbB2* transgene expression *in vivo*. (*a*) A germ cell-specific expression of EGFP in seminiferous tubules of the transgenic rat. (*b*) A subpopulation of spermatogonia expressing iDN-ERBB2 tagged with V5 molecular tag in the seminiferous tubules of transgenic rat after 4-HO-tamoxifen induction via PI injection. (*c*) An overlay of EGFP and V5 staining

we expect this animal to be a valuable resource for further investigation of ERBB receptor-mediated cell signaling at molecular levels both *in vivo* and *in vitro*.

CONCLUSIONS

Clonally derived spermatogonial stem cell lines that express an inducible dominant negative form of ERBB2 demonstrated an obvious difference in morphological appearance and growth curve of induced colonies comparing to uninduced ones. Interference with the normal function of ERBB2 leads to a slowdown in cell proliferation. This conclusion correlates with the results obtained by other reseachers [49, 50]. The recombinant protein with truncated kinase domain alters metabolic pathways involved in the regulation of cell division. Western blot analysis of induced and uninduced cells revealed significant differences in presence and phosphorylation state of some important proteins involved in ERBB2 mediated signal transduction. Germ cell-specific Inducible dominant negative ERBB2 transgenic rat proved to be a valuable model as a source of cells for dissecting ERBB2 dependent pathways in spermatogonia *in vitro*.

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CONFLICT OF INTEREST STATEMENT

No potential conflicts of interest are disclosed.

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