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ORIGINAL ARTICLE

Janus kinase 2 is required for the initiation but not maintenance of prolactin-induced mammary cancer

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The prolactin receptor (PRLR), its associated Janus kinase 2 (Jak2) and the signal transducer and activator of transcription 5 (Stat5) are essential for normal mammary gland development. Owing to the upregulation of the PRLR and the local synthesis of its ligand in neoplastic cells, it has been proposed that PRL can act as a local growth factor in human breast cancers. This notion is supported by experimental evidence in transgenic mice, which showed that the mammary-specific expression of PRL contributes to carcinogenesis *in vivo*. To assess the importance of Jak2/Stat5 signaling during mammary cancer initiation and progression, we generated a PRL-induced mammary cancer model that allows the functional ablation of the *Jak2* gene in the mammary epithelium before and after neoplastic transformation. Collectively, the results of this study show that the functional ablation of Jak2 protects against the onset of PRL-induced mammary tumorigenesis, suggesting that targeting this kinase is a relevant strategy for mammary cancer prevention. Surprisingly, Jak2 deficiency did not affect the growth and survival of PRL-induced mammary cancer cells in culture and *in vivo*. Consequently, Jak2 cannot be a sole therapeutic target to treat the established disease. PRL-induced mammary cancers exhibited an upregulation of ErbB2 and other ErbB receptor tyrosine kinases that may supersede the functionality of PRLR signaling through Jak2.
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Introduction

The peptide hormone prolactin (PRL) has a pivotal role in the occurrence of breast cancer in humans and animal models. High circulating levels of PRL increase the risk of breast cancer in pre- and post-menopausal women (Hankinson *et al.*, 1999; Tworoger and Hankinson, 2006), and the PRL receptor (PRLR) is overexpressed in >95% of breast cancer cases (Clevenger *et al.*, 1995; Ginsburg and Vonderhaar, 1995). The examination of transgenic mouse models provided experimental evidence that a sustained increase in the levels of circulating lactogenic hormones, in particular PRL, causes mammary cancer (Tornell *et al.*, 1991; Wennbo *et al.*, 1997). More recently, it has been shown that human breast cancer cells are able to upregulate the local synthesis of PRL, suggesting that this hormone can act in an autocrine manner to promote the proliferation of neoplastic cells (Clevenger *et al.*, 1995; Ginsburg and Vonderhaar, 1995). This novel paradigm was verified in a transgenic model that overexpresses PRL in the mammary epithelium under the regulation of the PRL- and estrogen-unresponsive, neu-related lipocalin (NRL) promoter (Rose-Hellekant *et al.*, 2003). More than 65% of NRL-PRL transgenic females were reported to develop both estrogen receptor (ER)-positive and ER-negative lesions after a mean latency of 15 months.

In normal and neoplastic mammary epithelial cells (MECs), PRLR signaling synchronously activates multiple signaling cascades, such as the Janus kinase 2 (Jak2), the signal transducer and activator of transcription 5 (Stat5a and Stat5b), the Src kinase, mitogen-activated protein kinases and the phosphoinositide 3 kinase pathway (for a reference, see Wagner and Rui (2008)). Jak2 is essential for the tyrosine phosphorylation and activation of Stat5 in response to PRL signaling (Shillingford *et al.*, 2002; Wagner *et al.*, 2004). The phenotypic abnormalities observed in mammary glands of conventional and conditional knockout mice that lack PRL, its receptor or both Stat5 isoforms (that is, Stat5a and Stat5b) are strikingly similar (Horseman *et al.*, 1997; Ormandy *et al.*, 1997; Teglund *et al.*, 1998; Cui *et al.*, 2004; Wagner *et al.*, 2004). This suggests that the Jak2/Stat5 signaling cascade is crucial for mediating the main biological responses that are being initiated by PRL. Collectively, the murine knockout models showed that PRLR signaling through Jak2 and Stat5 is required for the proliferation and differentiation of alveolar progenitors during pregnancy and lactation.

Signal transducer and activator of transcription 5 has been shown to be activated (that is, phosphorylated on tyrosine residues 694 or 699 in Stat5a and Stat5b) in a subset of human breast cancer cases (Cotarla *et al.*, 2004; Nevalainen *et al.*, 2004). Similar to an increase in

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circulating levels of PRL or stimulating the local synthesis of this hormone in the mammary gland, the overexpression of wild-type Stat5a as well as a constitutively active Jak2/Stat5 fusion protein is sufficient to induce neoplastic transformation of MECs *in vivo* (Iavnilovitch *et al*., 2002, 2004). This finding supports the notion that a hyperactive Jak2/Stat5 signaling cascade might mediate the initiation of mammary cancer in response to elevated levels of PRL.

Despite the well-defined role of PRL signaling during the initiation of neoplastic transformation, the importance of this hormone, its receptor as well as downstream signaling mediators has never been examined in established mammary cancers using genetic model systems (Wagner and Rui, 2008). To assess specifically the significance of Jak2/Stat5 signaling in PRL-induced mammary carcinogenesis, we generated a mouse model that overexpresses PRL in the mammary epithelium that is conditionally deficient in Jak2. Using Cre-mediated recombination, we deleted the *Jak2* gene before or after neoplastic transformation. This unique approach allowed us to discriminate the role of the Jak2/Stat5 pathway during the initiation of mammary neoplasia, as well as the growth and survival of fully neoplastic mammary cancer cells *in vitro* and *in vivo*.

Results

Jak2 deficiency prevents the initiation of PRL-induced mammary cancer

Janus kinase 2 and its downstream effector Stat5 are essential mediators for the main biological functions of PRL in normal MECs. To study whether Jak2 and active Stat5 are also required for the initiation of PRL-induced mammary cancer, we generated a mouse model that overexpresses PRL locally in the mammary epithelium of females that are conditionally deficient in Jak2 (NRL-PRL MMTV-Cre $Jak2^{fl/fl}$). In this study, we used two NRL-PRL transgenic lines (1647-13 and 1655-8) that overexpress the rat PRL under the NRL promoter, the activation of which in the mammary epithelium is unresponsive to estrogen and PRL (Rose-Hellekant *et al*., 2003). We have shown previously that the mouse mammary tumor virus (MMTV)-Cre transgene is expressed throughout the entire mammary ductal network in both luminal and myoepithelial cells before puberty (Wagner *et al*., 2001). The MMTV-Cre-mediated conditional deletion of the *Jak2* gene does not significantly impair ductal elongation, but the lack of this kinase prevents the specification of alveolar buds located at the terminal ends of the ductal tree in nulliparous females (Wagner *et al*., 2004). About one-third of all NRL-PRL MMTV-Cre $Jak2^{fl/fl}$ females and their littermate controls expressing Jak2 (NRL-PRL $Jak2^{fl/fl}$) were bred at least once during their lifetime. As expected, females that were conditionally deficient in Jak2 were unable to nurse their offspring, regardless of the local synthesis of exogenous PRL in the mammary epithelium. Experimental animals and their controls were monitored twice weekly over a period of 24 months for the growth of mammary tumors. As illustrated in Table 1, approximately 27% of all control females developed palpable lesions after a mean latency of 21.5 (±2.1 s.d.) months. The reproductive status did not have a noticeable influence on the occurrence or latency of mammary cancer (not shown). In contrast to the controls, not a single Jak2 knockout female developed a palpable mammary tumor within the experimental time line. In addition to the primary mammary cancers in control mice, we collected mammary glands from Jak2-deficient and control females at the experimental end point, that is, the day of tumor resection in a subset of control females or after 24 months in tumor-free animals. The whole-mount analysis of mammary glands from animals that did not develop palpable cancers revealed that approximately 34% of the control tissues of both NRL-PRL lines contained at least one atypical hyperplastic lesion (Figures 1a and c, arrow). With the exception of two females of NRL-PRL line 1655-8, none of the Jak2-deficient mice exhibited any microscopic lesions (Figures 1b and d). Interestingly, the two small hyperplastic lesions of approximately 3 mm in diameter that were observed in the Jak2-deficient mice were located in close proximity to the nipples and not within distant ducts (Supplementary Figure S1). We have

Table 1 Tumor incidence in the females of two NRL-PRL transgenic lines that are conditionally deficient in Jak2 (MMTV-Cre $Jak2^{fl/fl}$) and their littermate controls expressing Jak2 ($Jak2^{fl/fl}$)

Genotype	*Number of females*	*Incidence of proliferative lesions*		
		Mammary tumors	*Atypical hyperplasia*	*Total*
Total				
$Jak2^{fl/fl}$	59	16 (27.1%)	20 (33.9%)	36 (61.0%)
MMTV-Cre $Jak2^{fl/fl}$	28	0	2 (7.1%)	2 (7.1%)
NRL-PRL (line 1647-13)				
$Jak2^{fl/fl}$	32	7 (21.9%)	13 (40.6%)	20 (62.5%)
MMTV-Cre $Jak2^{fl/fl}$	14	0	0	0
NRL-PRL (line 1655-8)				
$Jak2^{fl/fl}$	27	9 (33.3%)	7 (25.9%)	16 (59.3%)
MMTV-Cre $Jak2^{fl/fl}$	14	0	2 (14.3%)	2 (14.3%)

Abbreviation: NRL-PRL, neu-related lipocalin-prolactin.

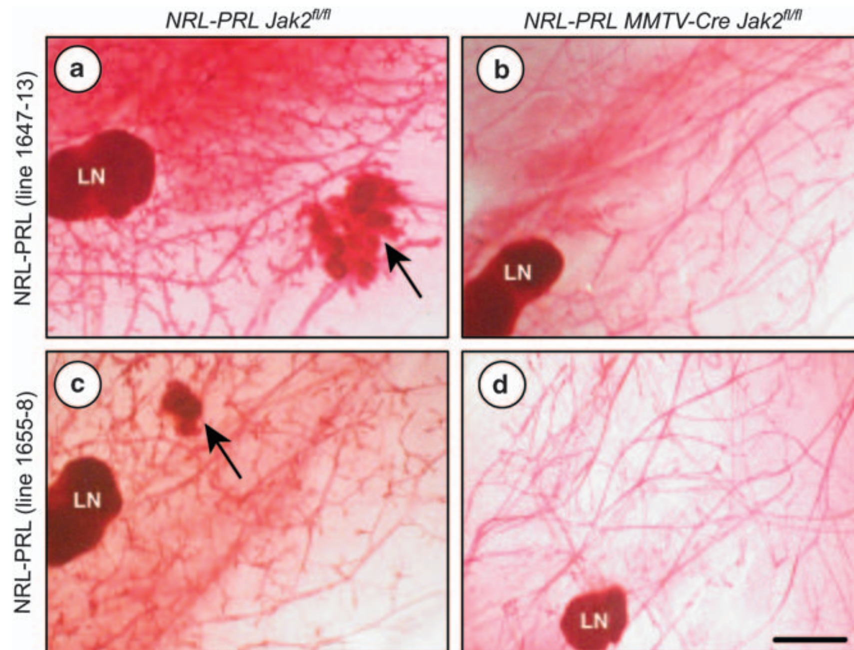

Figure 1 Inhibition of PRL-induced mammary tumorigenesis through deletion of *Jak2*. Mammary gland whole mounts from two NRL-PRL transgenic lines (1655-8 and 1647-13) that are Jak2-deficient (**b**, **d**) and their littermate wild-type controls (**a**, **c**). Arrows in (**a**) and (**c**) indicate the location of atypical hyperplastic lesions; LN, lymph node; bar = 1 mm.

Table 2 Expression of the ERα in PRL-induced mammary cancers

	Number of stained tumors	*ERα positive*		*ERα negative*
		Homogeneous	*Mixed*	
Total	16	2 (12.5%)	7 (43.8%)	7 (43.8%)
NRL-PRL (line 1647-13)	7	1 (14.3%)	3 (42.9%)	3 (42.9%)
NRL-PRL (line 1655-8)	9	1 (11.1%)	4 (44.4%)	4 (44.4%)

Abbreviations: ERα, estrogen receptor-α; NRL-PRL, neu-related lipocalin-prolactin.

previously reported that besides a lack of alveolar budding, Jak2-deficient mammary ducts were thinner and exhibited less tertiary branching (Wagner *et al*., 2004). These phenotypic abnormalities were also present in Jak2-deficient mammary glands overexpressing exogenous PRL in both NRL-PRL transgenic lines, suggesting that the lack of Jak2 effectively blocks the developmental effects of excess levels of locally synthesized PRL (Figures 1b and d). This might explain why Jak2-deficient females did not develop any palpable tumors and significantly less hyperplastic lesions within epithelial ducts.

It is generally known that the vast majority of genetically engineered mouse models for breast cancer that were generated over the past two decades develop ERα-negative mammary tumors. NRL-PRL transgenic females appear to be distinct from these earlier models, as they were reported to give rise to both ERα-positive and ERα-negative mammary cancers (Rose-Hellekant *et al*., 2003). In addition to a histological examination, we stained 16 mammary tumors from both NRL-PRL lines (that is, NRL-PRL (line 1647-13 or 1655-8) *Jak2*$^{fl/fl}$ control females) with an antibody against ERα to assess whether this steroid receptor was expressed (Table 2). We discriminated ERα-negative mammary tumors from neoplasia that exhibited ERα staining in more than 10% of the cancer cells, which are typically defined as ERα-positive tumors. To indicate the degree of intra-tumor heterogeneity between the various cancers, we further stratified tumors that exhibited a more homogeneous expression of ERα throughout the sections from those that were clearly comprised of both ERα-positive and ERα-negative cancer cells. ERα was present within nearly all nuclei in only two out of 16 primary cancers, and these mammary lesions were comprised of neoplastic cells that appeared more differentiated (Figure 2). Approximately 43% of all mammary cancers did not contain any epithelial cells that expressed ERα, and the remaining tumors (about 43%) exhibited mixed lesions that were comprised of ERα-negative epithelial cells containing regions that were clearly ERα positive. The ERα staining pattern among individual mammary tumors was indistinguishable between both NRL-PRL transgenic lines.

Jak2 is not required for the growth and survival of PRL-induced mammary cancer cells

As both the PRL receptor and its ligand are upregulated in human breast cancers, targeting the main downstream

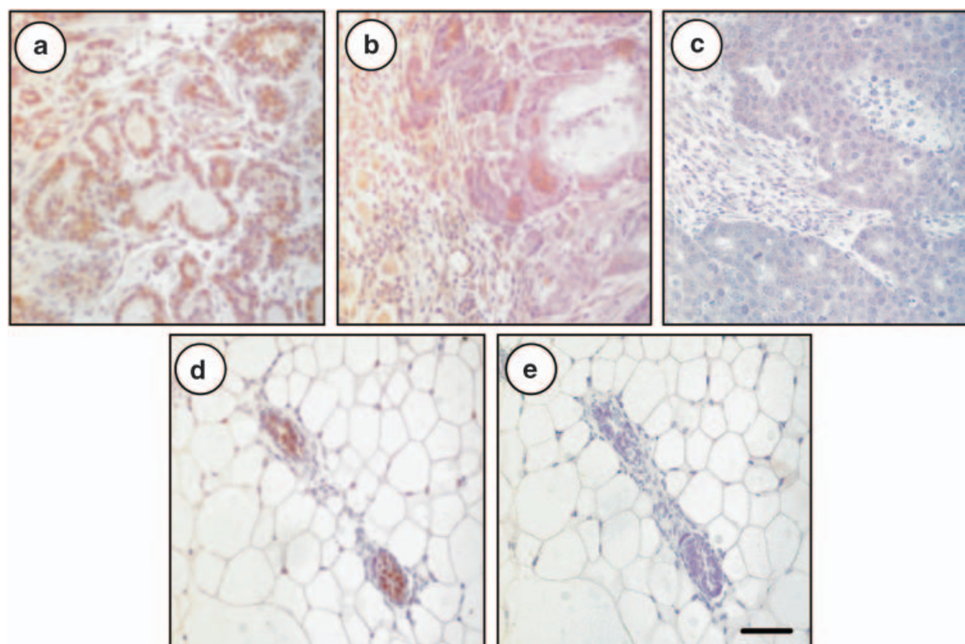

Figure 2 A subset of PRL-induced mammary cancers expresses the estrogen receptor-α (ERα). Immunohistochemistry of ERα in various mammary tumors (**a**–**c**) and the normal mammary epithelium (**d**). (**a**) ERα-positive tumor, (**b**) mixed lesion containing a subset of ERα-positive cells and (**c**) ERα-negative cancer. (**e**) A serial section of the mammary duct shown in panel d that was stained without the primary antibody against ERα. All slides were counterstained with hematoxylin (bar, 50 μm).

mediators of this hormone receptor was suggested to be a suitable strategy to treat breast cancer patients. As we have shown that Jak2 is essential for the onset of PRL-induced mammary cancers, a rational objective of our subsequent work was to address whether Jak2 and active Stat5 are required for the growth and survival of PRL-induced mammary cancer cells. To address this issue experimentally, we derived neoplastic cells from primary tumors of NRL-PRL *Jak2*$^{fl/fl}$ control females, and deleted the *Jak2* gene from these mammary cancer cells using an adenovirus expressing a Cre/green fluorescent protein (GFP) fusion protein (Figure 3a). Following the fluorescence-activated sorting of infected cells that were transiently positive for GFP, we obtained isogenic pairs of cancer cells with and without Jak2. The correct Cre-mediated excision of both floxed *Jak2* alleles was verified by polymerase chain reaction (PCR) and western blot analysis (Figures 3b and c). Notably, the isogenic pair of PRL-induced mammary cancer cells with and without Jak2 shown in Figure 3c expressed moderate levels of ERα. Despite the fact that the primary cancer cells were derived from PRL-induced tumors, they all lacked expression of active Stat5. This might suggest that most cancer cells had lost the PRL-mediated autocrine activation of its downstream mediator Stat5 in the progressing lesions (Figure 3d). Therefore, the deletion of Jak2 had no noticeable effect on the growth and survival of these cancer cells. Stimulation of the primary cancer cells with PRL was sufficient to induce the phosphorylation of Stat5. This clearly showed that these cells were still responsive to exogenous PRL. The deletion of *Jak2* led to a complete inhibition of Stat5 activation in these cells following PRL stimulation. In contrast to Stat5, loss of Jak2 had no significant effect on the expression and activation of Stat3, which was constitutively phosphorylated. Collectively, the successful generation of Jak2-deficient mammary cancer cells suggests that neither Jak2 nor active Stat5 are required for the growth- and survival-transformed MECs in culture.

Jak2 is dispensable for the engraftment and growth of cancer-initiating cells in vivo

The establishment of secondary cancers in recipient mice following transplantation serves as biological evidence that tumor-initiating cells that were cultured *ex vivo* still maintained their neoplastic characteristics. To address whether Jak2 is required in cancer-initiating cells *in vivo*, we transplanted 1×10^6 tumor cells with and without Jak2 into the collateral number 4 mammary glands of 20 athymic nude mice (that is, 10 mice or 20 transplants per cell line) and obtained 19 palpable tumors in total (that is, 10 with and nine without Jak2). As shown in Figure 4a, the functional ablation of Jak2 did not significantly alter the engraftment of cancer cells or the growth of the resulting tumor in recipient females. The subsequent analysis of genomic DNA from the Jak2-deficient tumors and their Jak2-expressing controls confirmed that all secondary lesions were comprised of the transplanted cancer cells carrying either two *Jak2* null alleles or two unrecombined *Jak2* floxed alleles in the controls (Figure 4b). Therefore, all secondary tumors arose solely from *Jak2*$^{-/-}$ cancer-initiating cells, suggesting that Jak2 is dispensable for the survival and numeric expansion of this progenitor pool. A *Jak2* wild-type allele served as an internal control in the PCR assay

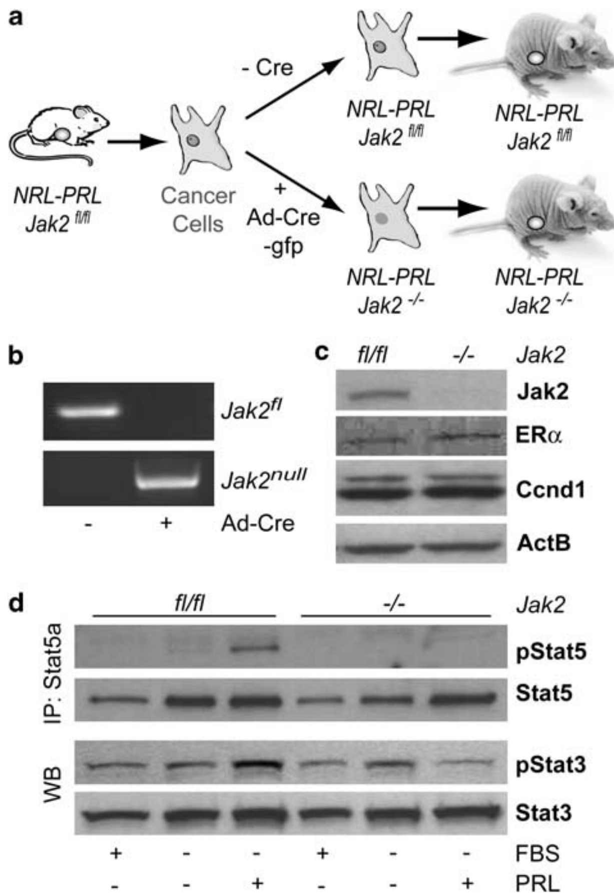

Figure 3 PRL-induced mammary cancer cells that are deficient in Jak2 lack activation of Stat5. (**a**) Experimental design for the conditional deletion of *Jak2* in PRL-induced mammary cancer cells. (**b**) PCR assay to verify the loss of both *Jak2* floxed alleles ($Jak2^{fl/fl}$) and the presence of *Jak2* knockout alleles ($Jak2^{-/-}$) following Cre-mediated recombination. (**c**) Expression analysis of Jak2, estrogen receptor-α (ERα) and cyclin D1 (Ccnd1) in Jak2-deficient cancer cells and their isogenic control cells. β-Actin (ActB) served as a loading control. (**d**) Immunoprecipitation (IP) and western blot (WB) analysis were used to assess the activation of Stat5 and Stat3 following PRL stimulation.

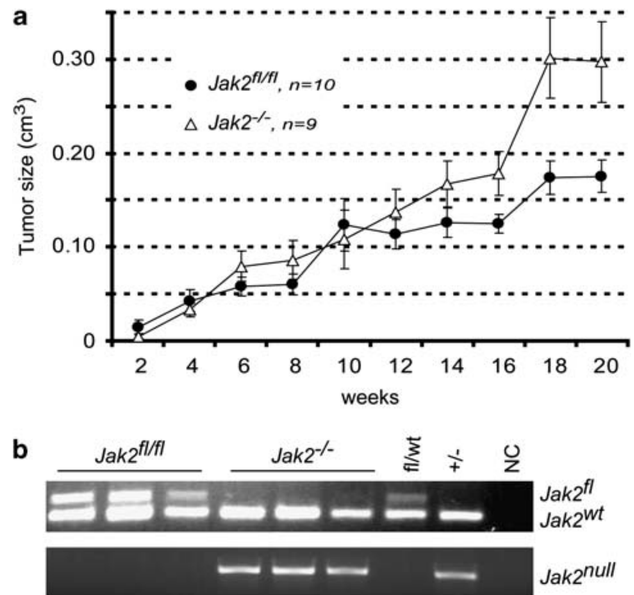

Figure 4 Jak2 deficiency does not affect the growth of PRL-induced mammary cancer cells *in vivo*. (**a**) Growth of PRL-induced mammary cancer cells lacking Jak2 and their isogenic controls in an orthotopic transplant of athymic nude mice. (**b**) PCR assay to verify the presence of *Jak2* floxed alleles or *Jak2* null alleles in resulting mammary tumors following orthotopic transplantation. Note that the *Jak2* wild-type allele ($Jak2^{wt}$), which was present in the PCR assay in both experimental groups, originated from stromal cells and blood vessels of the wild-type host. This allele was not present in the engrafted $Jak2^{fl/fl}$ or $Jak2^{-/-}$ cancer cells. NC, negative control.

shown in Figure 4b. This allele is not present in the transplanted cell types (that is, $Jak2^{fl/fl}$ or $Jak2^{-/-}$) and therefore originated from tumor-associated stromal cells and blood vessels of the wild-type host. The initial examination of secondary tumors revealed that, regardless of whether Jak2 was expressed or not, cancer cells lacked expression of active Stat5 (Figure 5a, upper panels). We therefore administered supraphysiological levels of PRL to a subset of recipient mice 30 min before collecting the tumor specimen to monitor the acute activation of Stat5 in response to PRL stimulation (Figure 5a, lower panels). Although normal MECs within the thoracic no. 3 mammary glands of recipient mice showed more extensive nuclear Stat5 staining in both experimental groups (Figure 5a, insets), the administration of PRL did not significantly increase the number of cells with active Stat5 in $Jak2^{fl/fl}$ control tumors, and nuclear Stat5 was not observed in $Jak2^{-/-}$ cancer cells. Collectively, these observations suggest that neither Jak2 nor active Stat5 are required for cancer cell growth and survival *in vivo*. Interestingly, the functional ablation of Jak2 did not affect the presence of ERα-positive cells within secondary lesions (Figure 5b). This might suggest that treatment of primary breast cancers with a Jak2 inhibitor will not lead to a selective expansion of ERα-negative cancer-initiating cells.

PRL-induced mammary cancers overexpress ErbB family members, which may supersede the functionality of Jak2

We have reported previously that the functional ablation of Jak2 in normal MECs and Jak2 conditional knockout mice results in a decrease in active Akt1, as well as expression and nuclear accumulation of cyclin D1 (Sakamoto *et al*., 2007). In contrast to our earlier studies in untransformed cells, Jak2 deficiency had no significant effect on the expression of Akt1 and cyclin D1 in PRL-induced mammary cancer cells *in vitro* (Figure 3c) and *in vivo* (Figure 5b, lower panel; Supplementary Figure S2). As Stat5 is not phosphorylated in these mammary cancer cells by receptor tyrosine kinases, cytokine receptor-associated Jaks other than Jak2, or cytoplasmic kinases, it is evident that compensatory signaling networks are being activated in neoplastic cells that supersede the function of Jak2 in regulating the expression of Akt1 and cyclin D1. ErbB receptor tyrosine kinases, which are upregulated or constitutively active in a significant subset of human breast cancer cases, are known to activate Akt1 and

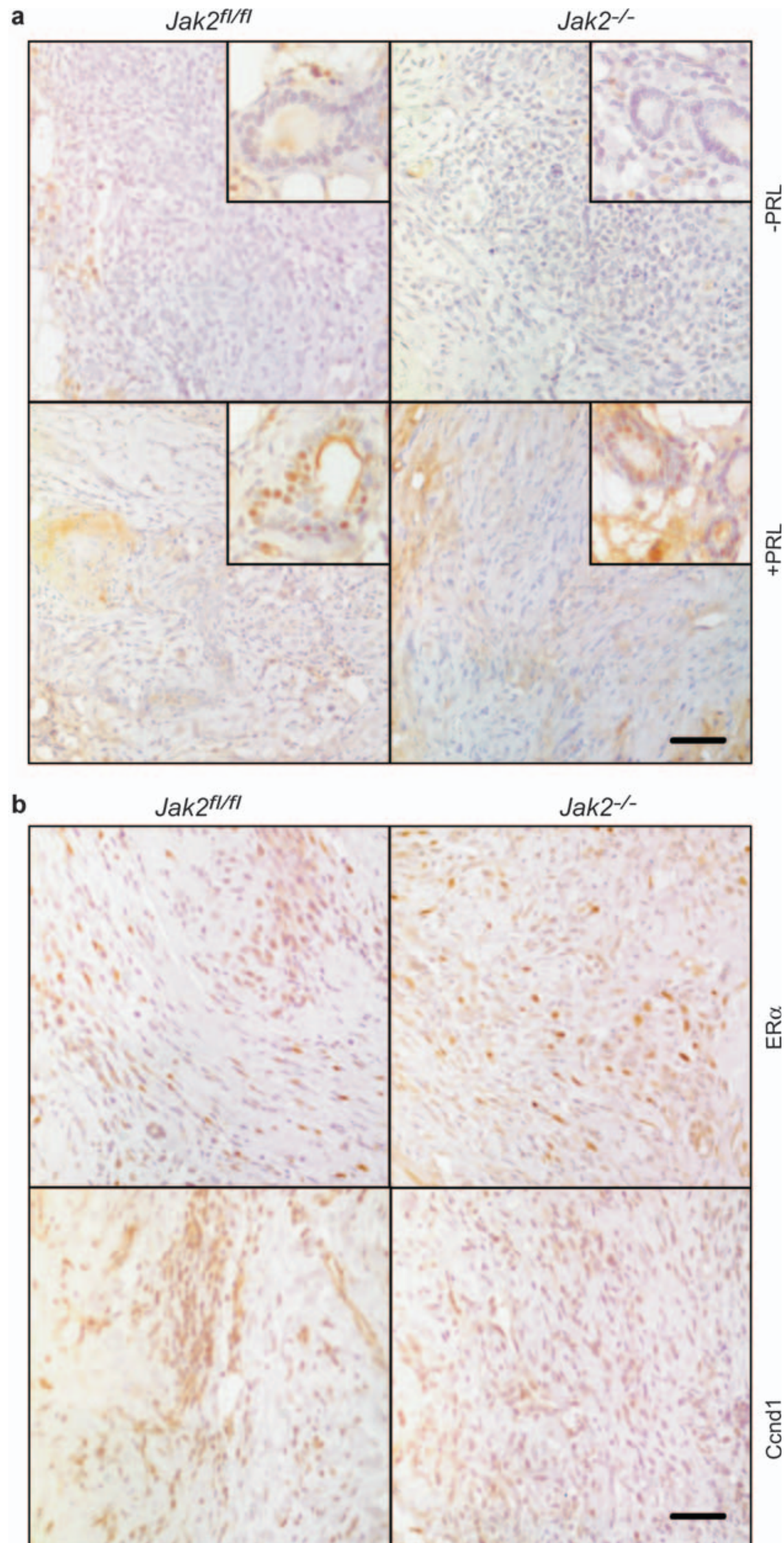

Figure 5 Jak2 deficiency leads to the lack of Stat5 activation, but not the expression of ERα and cyclin D1. (**a**) Immunohistochemistry was used to assess the PRL-mediated activation of Stat5 in control tumors and cancers lacking Jak2. Insets show the PRL-induced activation of Stat5 in normal no. 3 mammary glands derived from the tumor-bearing, wild-type recipient females. (**b**) Immunohistochemistry was used to determine the continuous expression of ERα and cyclin D1 (Ccnd1) in PRL-induced mammary cancers that lack Jak2 and their wild-type controls. All slides were counterstained with hematoxylin (bars, 50 μm).

Table 3 Expression of the ErbB family members in PRL-induced mammary cancers

	Number of stained tumors	*EGFR*	*ErbB2*	*ErbB3*	*ErbB4*
Total	16	4 (25.0%)	16 (100%)	7 (43.8%)	9 (56.3%)
NRL-PRL (line 1647-13)	7	3 (42.9%)	7 (100%)	4 (42.9%)	4 (57.1%)
NRL-PRL (line 1655-8)	9	1 (11.1%)	9 (100%)	3 (33.3%)	5 (55.5%)

Abbreviations: EGFR, epidermal growth factor receptor; NRL-PRL, neu-related lipocalin-prolactin.

thereby maintain the nuclear retention of cyclin D1 through inactivation of glycogen synthase kinase 3β (Diehl *et al*., 1998). We therefore assessed by western blot analysis and immunohistochemistry whether selected ErbB receptor tyrosine kinases were upregulated in PRL-induced mammary tumors. It is evident from the results shown in Table 3, Figure 6a and Supplementary Figure S3 that PRL-induced mammary cancers predominantly expressed ErbB2 compared with normal mammary gland tissues. In addition, other ErbB family members were upregulated in selective tumor specimens. In some cases, we detected a significantly elevated expression of all four ErbB family members (for example, Figure 6a, tumor no. 1). The ErbB2 receptor and its heterodimeric partner ErbB3 have been suggested to function as an oncogenic unit in breast cancer (Holbro *et al*., 2003; Kim *et al*., 2005). Despite variable expression of both receptors in individual PRL-induced mammary tumors, ErbB2 and ErbB3 engaged in heterodimer formation and were tyrosine phosphorylated (Figure 6b). As only Jak2-expressing females developed PRL-induced mammary cancer (that is, NRL-PRL *Jak2*$^{fl/fl}$), it is apparent that the selective upregulation of these receptor tyrosine kinases occurs independently of Jak2/Stat5 signaling during or following neoplastic transformation. In addition, the reduction in the functionality of Jak2 and therefore loss of nuclear Stat5 during disease progression seem not to have an effect on the elevated expression levels of ErbB kinases (Figure 6c). Interestingly, Jak2 deficiency in normal MECs in culture already seems to favor the selection of cells with an upregulated expression of the epidermal growth factor receptor (Figure 6c), and it is therefore possible that epithelial subtypes with high ErbB activity might be the cancer-initiating population in NRL-PRL transgenic females. Collectively, the results of this study show that the upregulation of ErbB receptor tyrosine kinase might be sufficient to supersede the importance of Jak2/Stat5 signaling in cancer cells. This might explain why targeting Jak2 alone is not sufficient to halt the growth and survival of mammary cancer cells in culture and *in vivo*.

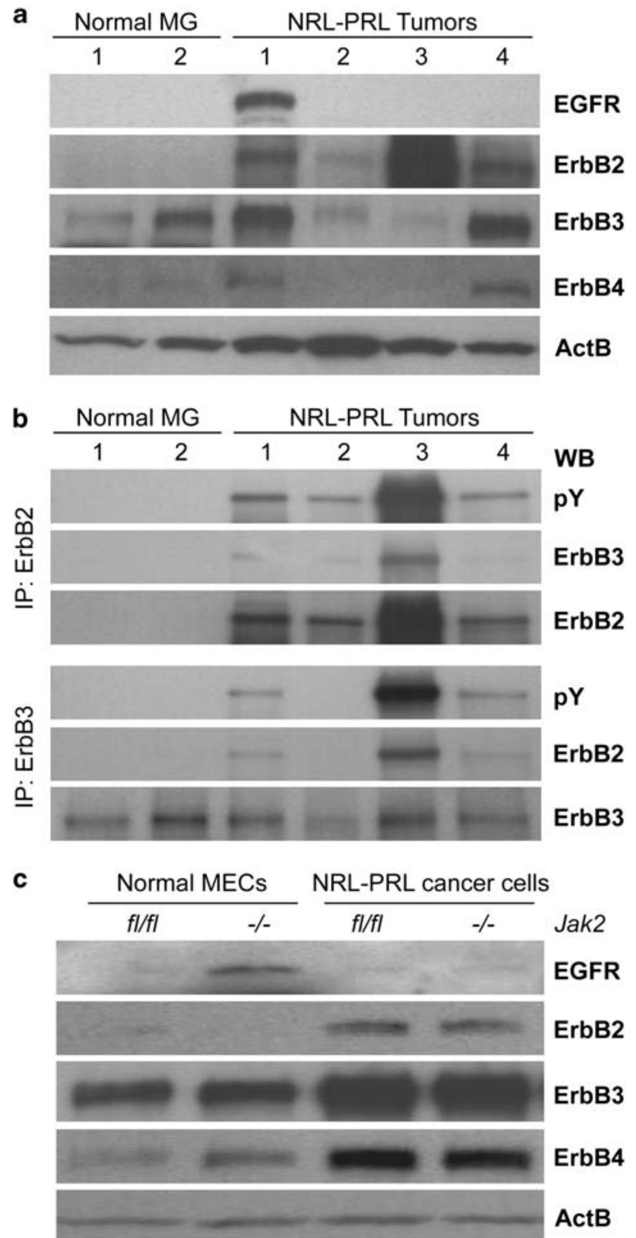

Figure 6 PRL-induced mammary cancers exhibit an upregulation of ErbB receptor tyrosine kinases. (**a**) Western blot analysis was used to determine the expression of all four ErbB family members in NRL-PRL-induced mammary cancers as well as normal mammary glands (MG) from lactating NRL-PRL females. (**b**) Immunoprecipitation (IP) and western blot (WB) analysis were used to assess the tyrosine phosphorylation and heterodimer formation of ErbB2 and ErbB3. (**c**) Western blot analysis was used to assess the expression of ErbB receptors in untransformed and neoplastic mammary epithelial cells that lack Jak2 and their isogenic controls expressing Jak2. β-Actin (ActB) served as a loading control in both panels.

Discussion

The essential biological functions of the peptide hormone PRL during normal mammary gland development are mediated by Jak2 and its downstream effector Stat5. The functional ablation of Jak2 in the normal mammary epithelium before tumorigenesis clearly shows that this receptor-associated kinase is equally

important for mammary carcinogenesis in response to an increase in PRL signaling. Among various epithelial subtypes that originate during mammary gland development, alveolar progenitors are highly responsive to PRL, and it can be assumed that these cells are also the cellular targets for PRL-induced neoplastic transformation. We have previously reported that alveolar progenitors in nulliparous mice and parity-induced mammary epithelial cells (PI-MECs) in parous females facilitate mammary tumorigenesis in transgenic mice overexpressing wild-type ErbB2 (Henry *et al*., 2004). These cell types are located within lobular units, and they are responsive to pregnancy hormones. Although PI-MECs have a limited role as alveolar progenitors in multiparous females, these cells retain characteristics of multipotent stem cells, such as self-renewal and contribution to ductal and alveolar morphogenesis upon transplantation (Wagner *et al*., 2002; Boulanger *et al*., 2005; Matulka *et al*., 2007). Given the fact that the genesis of PI-MECs during pregnancy depends on PRL signaling, we anticipated that targeting Jak2 or Stat5 might be a feasible strategy to prevent the onset of PRL-mediated mammary cancer (Wagner and Rui, 2008). Indirect confirmation for this assumption was previously provided by studies involving mice that overexpress transforming growth factor-α and the SV40 large T-antigen. In these models, Stat5a or PRLR deficiency caused a delay or reduced incidence in mammary tumorigenesis (Humphreys and Hennighausen, 1999; Ren *et al*., 2002; Oakes *et al*., 2007; Miermont *et al*., 2010). The complete absence of PRL-induced mammary cancer in females lacking Jak2 now provides direct experimental evidence that targeting Jak2 might be a suitable strategy for the prevention of breast cancer subtypes that originate from luminal cells within terminal ducts and alveolar units that are responsive to PRL. Specifically, individuals with hyperprolactinemia or women who are at risk of developing pregnancy-associated breast cancers might benefit from such a preventive regimen.

Although the findings from various genetically engineered mouse models, including Jak2-deficient mice, suggest that PRL signaling through the Jak2/Stat5 cascade can contribute to mammary cancer initiation, *in vivo* model systems that specifically address whether inhibiting the activation of the PRLR or its downstream mediators is also a therapeutically relevant strategy to treat the established disease were missing. Multiple Jak2 tyrosine kinase inhibitors are currently being developed and tested to treat myeloproliferative disorders and other hematopoietic malignancies (for a review, see Pardanani, 2007), and it is therefore feasible that these drugs could also be used in therapeutic regimens to combat advanced breast cancer. Prior to such clinical studies, the availability of a Jak2 conditional knockout model can provide a unique opportunity to examine the significance of the Jak2/Stat5 signaling cascade in fully neoplastic mammary cancer cells that were transformed in response to an increase in PRL signaling. Although Jak2 is essential for the onset of PRL-induced mammary tumorigenesis, the deletion of *Jak2* following neoplastic transformation had no significant impact on the survival and growth of mammary cancer cells in culture and *in vivo*. These observations clearly suggest that Jak2 cannot be the sole target to treat advanced breast cancers that are of luminal origin and that express the PRLR. Our findings are in line with observations in primary human breast cancer cases, which show that transcriptionally active Stat5 is not required for cancer progression and metastasis. Nuclear Stat5 was reported to contribute to a more differentiated phenotype of breast cancer cells, and it might therefore serve as a favorable prognostic marker, in particular for lymph node-negative breast cancers (Nevalainen *et al*., 2004). Among both Stat5 isoforms, Stat5a has the predominant role in promoting the differentiation and in suppressing the motility of cancer cells, and this effect might be facilitated through suppression of Bcl6 (Tang *et al*., 2010; Tran *et al*., 2010).

At this point, we cannot exclude that PRLR signaling is still capable of promoting breast cancer progression and invasion through Jak2/Stat5-independent pathways such as c-Src, FAK and mitogen-activated protein kinases. We and others have shown recently that the activity of c-Src does not depend on the functionality of Jak2 as suggested previously (Dominguez-Caceres *et al*., 2004; Sakamoto *et al*., 2007; Garcia-Martinez *et al*., 2010). The contribution of these signal transducers during PRL-induced mammary tumor progression needs to be examined in more detail using novel *in vivo* model systems that allow a conditional inactivation of signal transducers, such as c-Src. It is evident that conventional knockout models (for example, cyclin D1, c-Src or Akt1-deficient mice) in combination with oncogene-expressing transgenics are not suitable to address specifically whether the targeted inhibition of signal transducers is therapeutically relevant (Matulka and Wagner, 2005). Many of these animals lack expression of putative therapeutic targets from the day they were conceived and never develop mammary cancer. Consequently, they can only serve as models for cancer prevention and not for therapy. Our studies in females that are conditionally deficient in Jak2 clearly show that the significance of particular signal transducers can shift dramatically following neoplastic transformation. Cancer cells frequently upregulate or acquire mutations within receptors or associated kinases that mediate self-sufficiency in growth signals and evasion from apoptosis. In particular, receptor tyrosine kinases such as ErbB2 are upregulated in a significant subset of human breast cancers (Slamon *et al*., 1989), and these kinases phosphorylate the same downstream signaling mediators that are being activated by multiple cytokine receptors, including the PRLR. This might explain why PRL potentiates signals downstream of other growth factor receptors and why the overexpression of ErbB receptors and their ligands such as transforming growth factor-α is able to cooperate with PRLR signaling during tumor initiation (Arendt *et al*., 2006; Arendt and Schuler, 2008). In this study, we observed that PRL-induced mammary tumors exhibited an upregulation of ErbB2 and selected other members of the ErbB family,

and these molecular changes may function independently of Jak2/Stat5 signaling. We propose that the gain-of-function of receptor tyrosine kinases during neoplastic transformation is able to supersede the importance of Jak2/Stat5 signaling in regulating downstream effectors following mammary cancer initiation. Although the PRL-induced activation of Jak2 and Stat5 is able to regulate the levels of active Akt1 and nuclear accumulation of cyclin D1 in normal cells (Brockman *et al*., 2002; Brockman and Schuler, 2005; Sakamoto *et al*., 2007), we have shown recently that the expression of constitutively active ErbB2 was sufficient to elevate the expression of these downstream mediators independently of Jak2. Similar to PRL-overexpressing mice, Jak2 has a role in the initiation of ErbB2-associated mammary tumorigenesis, but Jak2 is dispensable for the maintenance of ErbB2-expressing mammary cancer cells (Sakamoto *et al*., 2009). Although Jak2 may not be a sole target to treat the established disease, targeting this kinase to prevent the initiation of breast cancer might be more broadly applicable, as Jak2 is required for the proliferation of luminal progenitor cells located within terminal ducts and alveolar units that are prime targets for growth factor-induced neoplasia (Wellings *et al*., 1975; Cardiff, 1998). Besides, it has been proposed recently that luminal progenitors are the likely source of basal-like breast tumors in humans (Lim *et al*., 2009). Future studies will show whether Jak2 is also required for the genesis of breast cancer subtypes that express basal epithelial cell markers, such as Brca1-associated mammary tumors (Triplett *et al*., 2008).

Materials and methods

Mouse models

Mouse mammary tumor virus -Cre (Wagner *et al*., 1997a) transgenics were crossed with Jak2 conditional knockout mice. The generation of genetically engineered animals with *Jak2* conditional knockout alleles (*Jak2$^{fl/fl}$*) and the PCR protocols that were used to determine the presence of *Jak2* floxed, *Jak2* recombined/null and *Jak2* wild-type alleles have been described previously (Krempler *et al*., 2004; Wagner *et al*., 2004). In this study, we used two transgenic lines that overexpress PRL in the mammary gland under the control of the NRL promoter (NRL-PRL, lines 1647-13 and 1655-8) (Rose-Hellekant *et al*., 2003). All targeted alleles and transgenes were carried in a predominantly FvB background (>50–75%). To establish orthotopic transplant models, 1.0×10^6 mammary cancer cells deficient in Jak2 and isogenic wild-type control cells were injected into the number four inguinal mammary glands of athymic nude females (NCr strain; National Cancer Institute, Bethesda, MD, USA). Tumor volumes were measured every 2 weeks using a caliper. To estimate the tumor volume, we used the following equation as described previously (aoui-Jamali *et al*., 2003): volume $= \pi/6$ (length $\times$ width^2). Thirty minutes before the experimental end point and harvesting the tumors, a subset of mice was injected intraperitoneally with ovine PRL (AFP-10692C; 5 μg per gram of body weight) or saline as a control to assess the PRLR/Jak2-dependent activation of Stat5. All animals were treated humanely and in accordance with the institutional guidelines and federal regulations.

Cell culture

Mammary cancer cells were derived from tumor-bearing NRL-PRL Jak2$^{fl/fl}$ females and cultured according to a protocol published by Medina and Kittrell (2000). To delete both *Jak2* conditional knockout alleles, primary tumor cells were infected with an adenoviral vector expressing a fusion protein of Cre recombinase and GFP (Vector Biolabs, Burlingame, CA, USA) and fractionated using fluorescence-activated cell sorting. Recombinant mouse PRL (AFP306C) was kindly provided by Dr AF Parlow under the sponsorship of the National Hormone and Pituitary Program, NIDDK (National Institutes of Health, Bethesda, MD, USA). Mammary cancer cells were treated with 10 nM of PRL for 20 min to induce the activation of Stat5.

Immunoprecipitation and western blot analysis

The preparation of whole-cell extracts of clarified cell lysates and tissue homogenates, as well as the experimental procedures for immunoprecipitation and western blot analysis were described in detail elsewhere (Sakamoto *et al*., 2007). The following antibodies were used for immunoblotting: α-β-actin (I-19; 1:2000 dilution), α-cyclin D1 (72G-13; 1:1000 dilution), α-ERα (MC-20; 1:1000 dilution), α-ErbB3 (C-17; 1:1000 dilution) and α-ErbB4 (C-18; 1:1000 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); α-Stat5 (2:250 dilution) from BD Biosciences (San Jose, CA, USA); α-pAkt (Ser473; 1:1000 dilution), α-Akt (1:1000 dilution), α-pStat3 (Tyr705; 58E12; 1:1000 dilution) and α-Stat3 (124H6; 1:1000 dilution) from Cell Signaling Technology (Danvers, MA, USA); α-epidermal growth factor receptor (ab2430; 1:200 dilution) from Abcam (Cambridge, MA, USA); α-Jak2 antibody (691R5; 1:2000 dilution) from Biosource (Carlsbad, CA, USA); and α-ErbB2/c-Neu (Ab-3; 1:1000 dilution) from Calbiochem (Gibbstown, NJ, USA). The α-phospho-Stat5a/b (Y694/9) antibody (AX1; 2 μg/ml; Advantex Bioreagents, El Paso, TX, USA) was kindly provided by Dr H Rui (Thomas Jefferson University, Philadelphia, PA, USA). The polyclonal α-Stat5a antiserum was a gift from Dr L Hennighausen (National Institutes of Health).

Whole-mount staining and immunohistochemistry

Whole mounts were prepared and stained in carmine alum as described previously (Wagner *et al*., 1997b). Basic protocols for immunohistochemistry on paraffin-embedded mammary gland specimens were described elsewhere (Wagner *et al*., 2004). The following antibodies were used for immunohistochemistry: α-pStat5A/B (Tyr694/699) antibody (1:100 dilution) from Upstate Biotechnology (Billerica, MA, USA); α-ERα (1115-1; 1:100 dilution) from Epitomics (Burlingame, CA, USA); α-cyclin D1 antibody (Ab-4, 1:250 dilution) from NeoMarkers (Fremont, CA, USA); α-epidermal growth factor receptor (ab2430; 1:200 dilution) from Abcam; α-HER2/ErbB2 (1:25 dilution) from Cell Signaling Technology; α-ErbB3 (C-17; 1:100 dilution) and α-ErbB4 (C-18; 1:100 dilution) from Santa Cruz Biotechnology; α-CK14 antibody (1:1000 dilution) from Convance (Princeton, NJ, USA); and α-CK8 antibody (TROMA-I; 1:250 dilution) from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA, USA). For visualization of the specific targets, we used corresponding biotinylated secondary antibodies and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

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