Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2015 March 20.

Published in final edited form as:

Oncogene. 2015 March 19; 34(12): 1596–1607. doi:10.1038/onc.2014.53.

IL-13Rα2 mediates PNR-induced migration and metastasis in ERα-negative breast cancer

Z Zhao, **L Wang**, and **W Xu**

Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI, USA

Abstract

Emerging evidence has linked photoreceptor cell-specific nuclear receptor (PNR/NR2E3), an orphan nuclear hormone receptor, to human breast cancer. PNR was shown to be a transcriptional activator of estrogen receptor-α (ERα) in ERα-positive breast cancer cell lines and high-level expression of PNR correlates with favorable response of ERα-positive breast cancer patients to tamoxifen. Interestingly, gene expression microarray study shows that PNR regulates distinct genes from those regulated by ERα, suggesting that PNR could have ERα-independent functions. Herein, we investigated the function of PNR in ERα-negative breast cancer cells. Our results showed that PNR-induced cell migration and metastasis of ERα-negative breast cancer cells both *in vitro* and *in vivo*, and the effect was attributed to the upregulation of interleukin (IL)-13Rα2, a high-affinity receptor for IL-13 that regulates tumor growth, invasion and metastasis of various human cancers. Mechanistically, PNR activated transcription of IL-13Rα2 through direct recruitment to IL-13Rα2 promoter. Upon stimulation with IL-13, IL-13Rα2 increased the extracellular signal-regulated kinases 1 and 2 phosphorylation, which led to breast cancer migration and metastasis. The IL-13 triggered signal cascade was specific to IL-13Rα2, as the closely related IL-13Rα1 was not regulated by PNR. IL-13Rα2 is a novel tumor antigen that is overexpressed in a variety of solid tumor types. This study presents the first evidence that PNR could promote ERα-negative breast cancer metastasis through activation of IL-13Rα2-mediated signaling pathway.

INTRODUCTION

Photoreceptor cell-specific nuclear receptor (PNR), an orphan nuclear receptor, is highly expressed in retinal cells and has crucial roles in photoreceptor development, differentiation and survival.[1–3] Emerging evidence implicate roles of PNR in breast cancer.[4,5] Breast cancers are biochemically classified into three subtypes: estrogen receptor-α (ERα)-positive, Her2-positive and triple-negative breast cancers that lack the expression of ERα, progesterone receptor and Her2. In ERα-positive breast cancer cell lines, PNR directly binds

© 2014 Macmillan Publishers Limited All rights reserved

Correspondence: Professor W Xu, Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, 1400 University Avenue, Madison, WI 53706, USA. wxu@oncology.wisc.edu.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

to the promoter region of ERα and increases ERα expression.[4] By knocking down PNR or ERα individually in MCF7 cells, gene networks shared between PNR and ERα were identified using DNA microarray analysis.[4] Although 525 target genes were shared between PNR and ERα in MCF7 cells, 1847 target genes were solely regulated by PNR independent of ERα, indicating that PNR may have functions regardless of ERα expression (for example, in ERα-negative breast cancer).[4] In another study, the expression of all human nuclear receptors was examined in 116 human breast samples containing both normal and neoplastic breast tumors. PNR was found expressed in ERα-negative breast cancer.[5] Thus far, the role of PNR in ERα-negative breast cancer has not been investigated.

Interleukin-13 (IL-13), a T_H2 cytokine, has crucial roles not only in cancers but also in the autoimmune diseases.[6,7] IL-13 interacts with two membrane receptors, a heterodimeric receptor composed of IL-4Rα and IL-13Rα1 and monomeric IL-13Rα2 receptor. IL-13Rα1 reacts with both IL-4 and IL-13 and signals through activation of insulin receptor substrate-phosphatidylinositol 3 kinase-AKT and signal transducer and activator of transcription 6 pathways in macrophages.[8] IL-13Rα2, on the other hand, only reacts with IL-13 at high affinity. IL-13 has been shown to enhance extracellular signal-regulated kinases 1 and 2 (ERK1/2), activator protein 1 (AP-1) and matrix metalloproteinase (MMP) activities though IL-13Rα2 in ovarian cancer cells and regulate invasion and metastasis.[9–11] IL-13Rα2 has also been shown to mediate invasion and metastasis in pancreatic[10] and colorectal[9] cancers. Depending on cell types, the activation of IL-13Rα2 by IL-13 induced various downstream signaling pathways including ERK/AP-1, transforming growth factor-β1, phosphatidylinositol 3 kinase, AKT and Src activation.[7,9–11] AP-1 complex and signal transducer and activator of transcription 6 were reported as upstream transcriptional factors for IL-13Rα2 in glioma and keratinocytes, respectively.[12,13] IL-13Rα2 is highly expressed in various solid tumors and regarded as a novel tumor antigen.[14] Minn *et al.*[15] reported a positive correlation between IL-13Rα2 expression and breast cancer metastasis to lung. However, the function and regulation of IL-13Rα2 in breast cancer have not been fully elucidated.

In this study, we attempted to elucidate the role of PNR in ERα-negative breast cancer and unexpectedly discovered a novel functional crosstalk between PNR and IL-13/IL-13Rα2 in regulating tumor migration and metastasis. Although PNR did not affect cell proliferation *in vitro*, it enhanced migration and metastasis of ERα-negative cells *in vitro* and *in vivo*. PNR transcriptionally activated IL-13Rα2 by binding to the proximal promoter region of IL-13Rα2, which led to upregulation of IL-13Rα2 and activation of ERK1/2. Both PNR overexpression and IL-13 treatment increased cell migration, which could be abrogated by IL-13Rα2 knockdown. Moreover, IL-13 expression level inversely correlated with the overall survival of human breast cancer patients. Collectively, we have demonstrated that IL-13/IL-13Rα2 signaling has a pivotal role in breast cancer migration and metastasis, and this pathway is significantly enhanced by PNR in ERα-negative cells. Our results suggest that targeting IL-13 signaling in combination with inhibiting the activity of PNR may impose synergistic beneficial effects on triple-negative breast cancer patients.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

RESULTS

PNR enhances the migration and colony formation ability of LM2 cells

To investigate the function of PNR in ERα-negative breast cancer cells, we stably expressed either full-length PNR or FLAG-tagged full-length PNR (FLAG-PNR) in ERα-negative MDA-MB-231 and LM2 cell lines (Figure 1a). LM2 cell line was derived from MDA-MB-231 by *in vivo* bioluminescence selection with high tendency for lung metastasis.[15] As controls, we also generated ERα-positive MCF7 and T47D cell lines stably expressing PNR (Supplementary Figure S1A). FLAG-PNR exhibited nuclear localization by immunofluorescence using an α-FLAG antibody, in conforming to its role as a transcriptional factor (Figure 1b).[4,16–19] Further, we found that PNR overexpression did not alter the cell morphology of MDA-MB-231 and LM2 cell lines (Figure 1c), nor did it significantly affect cell proliferation *in vitro* (Figure 1d).

In order to examine the effect of PNR on cell migration, wound-healing assay was performed and quantified. Although PNR overexpression had no effect on migration of MCF7, T47D (Supplementary Figure S1B) and MDA-MB-231 cells (Figure 2a), it induced rapid wound closure in LM2 cells (Figure 2a). To quantitatively measure the increase of migration, Boyden chamber transwell assay was used where PNR overexpression was shown to increase migration of LM2 cells by ~1.6-fold (Figure 2b). Conversely, knocking down PNR by small interfering RNA reduced the endogenous PNR expression by 60% and decreased migration of LM2 cells (Figure 2c). Overexpression of PNR also increased the colony formation ability of LM2 cells under low-density seeding (Figure 2d), whereas it did not seem to alter cell adhesion as no obvious difference could be detected between green fluorescent protein (GFP) control cells and PNR-overexpressing cells with regards to their binding to fibronectin, laminin or collagen in the adhesion assay (Figure 2e). Collectively, PNR increased the migration and colony formation ability of LM2 cells, two parameters often measured *in vitro* that are indicative of metastatic potential *in vivo*.[20–22]

PNR promotes metastasis in the xenograft mouse models

As PNR enhanced migration of LM2 cells, we further investigated the role of PNR in tumorigenesis using the mammary fat pad xenograft mouse model. LM2 cells were retrovirally infected with GFP or PNR and injected into the inguinal mammary fat pads of nude mice. The tumor growth was measured with digital caliper over the course of experiment for 40 days. The mice injected with PNR-overexpressing LM2 cells developed tumors at a greater rate than the non-PNR-expressing control (Figures 3a and b). To verify that PNR remained to be expressed in tumors at the end of the study, the immunohistochemistry staining of PNR was performed. The strong nuclear staining of PNR remained to be detected after 40 days of *in vivo* tumor growth (Figure 3c). Pre-incubation of blocking peptides with anti-PNR antibody markedly diminished the nuclear staining, demonstrating that PNR antibody is target specific (Figure 3c). When the mice were dissected for histologically analysis, lung metastasis was detected in the PNR-overexpressing group (20%) but not in the GFP control group (0%). Hematoxylin and eosin staining showed that, in contrast to the GFP control group with no infiltrated tumor cells, lung of PNR group was heavily infiltrated with tumor cells (Figure 3d). To exclude the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

possibility of inflammation, we performed immunohistochemistry using antibodies against human Ki67 and firefly luciferase because LM2 cells stably express firefly luciferase when they were selected *in vivo*.[15] The nuclear staining of human Ki67 confirmed the human origin of the cells and the cytoplasmic staining of firefly luciferase verified the identity of LM2 cells[15] (Figure 3d).

IL-13Rα2 mediates PNR-induced migration of LM2 cells

The primary tumor outgrowths of breast cancer and lung metastasis are a multiple gene-regulated event.[23,24] The LM2 cell line was originally isolated from a lung metastasis of the parental MDA-MB-231 cell line and is known for its high tendency to metastasize to lung.[15] This prompted us to investigate whether PNR regulates genes involved in lung metastasis that may account for PNR-mediated migration and metastasis of LM2 cells. We first examined whether the expression of lung metastasis-related genes[15] was altered by overexpressing PNR. Among the 10 reported metastasis genes being examined, IL-13Rα2 mRNA level was strongly increased by PNR overexpression in MDA-MB-231 and LM2 cells (Figure 4). The upregulation of the expression of IL-13Rα2 mRNA by PNR was also observed in two additional triple-negative breast cancer cell lines, MDA-MB-468 and BT549 (data not shown). Although several other genes such as *MMP1* and *EFEMP-1* were also upregulated by PNR overexpression in LM2 cells (Figure 4b), the fold of activation was not as significant as IL-13Rα2 and the upregulation was only found in LM2 but not in the parental MDA-MB-231 cells.

As PNR overexpression increases IL-13Rα2 mRNA level in cell lines, we next investigated whether the expression of PNR and IL-13Rα2 positively correlates with each other in human breast tumors. First, a positive correlation between PNR and IL-13Rα2 mRNA levels ($r = 0.24$, $P<0.0001$; Figure 5a) was observed using the bc-GenExMiner database with a collection of 3161 breast cancer patients.[25] The correlation coefficient is comparable to that of PNR and ERα ($n = 2872$, $r = 0.26$, $P<0.0001$) (Supplementary Figure S2A), which has been shown to have positive correlation.[4] IL-13 signals through two membrane-bound receptors, IL-13Rα1 and IL-13Rα2.[8,26,27] IL-13Rα1, only upon heterodimerization with IL-4Rα, binds to IL-13 with relatively high affinity,[28–30] whereas IL-13Rα2 binds to IL-13 with ~ 50-fold higher affinity than IL-13Rα1.[31,32] No correlation at the mRNA levels could be found between PNR and IL-13Rα1 or with IL-4R (Supplementary Figure S2B). Consistently, IL-13Rα1 mRNA was found not regulated by PNR (Supplementary Figure S2C). Interestingly, positive correlation could be observed between PNR and IL-13 at the mRNA level (Supplementary Figure S2B). When the endogenous PNR was knocked down by small interfering RNA (Figure 2c), IL-13Rα2 mRNA level was decreased by ~ 60%, whereas IL-13Rα1 mRNA was not affected (Figure 5b). Notably, MMP1 mRNA level was also decreased by knocking down PNR (Figure 5b). This was most likely due to a secondary effect resulted from the decrease of IL-13Rα2 mRNA because IL-13Rα2 was shown to affect MMP family gene expression[11] and knockdown of IL-13Rα2 also caused the reduction of MMP1 mRNA levels (data not shown). Next, the role of IL-13Rα2 in regulating migration of breast cancer cells was analyzed. IL-13Rα2 was knocked down by two short hairpin RNAs expressing lentivirus specific for IL-13Rα2, A11 and E4, in LM2 cells. These short hairpin RNAs could effectively knockdown IL-13Rα2 as detected at both

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

mRNA and protein levels (Figure 5c), while not affecting IL-13Rα1 mRNA level (Supplementary Figure S2D). Although silencing of IL-13Rα2 in LM2 cells has no effect on cell proliferation (Figure 5d), it decreased migration as shown in the transwell assay (Figure 5e), which is opposite to the effect of PNR overexpression. Finally, to demonstrate whether PNR-induced cell migration is mediated through IL-13Rα2, we overexpressed PNR in LM2 cells, while simultaneously silencing IL-13Rα2. Knockdown of IL-13Rα2 abrogated the PNR-enhanced cell migration (Figure 5f). Together, these results suggest that PNR promotes the migration of LM2 cells via activation of IL-13Rα2 signaling.

IL-13 enhances ERK1/2 phosphorylation and regulates migration of LM2 cells in IL-13Rα2-dependent manner

IL-13 is a high-affinity ligand for IL-13Rα2 and IL-13 signaling has been implicated in tumor malignancy of pancreatic, ovarian and colorectal cancers.[6,9–11] To examine whether IL-13 regulates breast cancer cell migration and metastasis, we treated the control and PNR-overexpressing LM2 cells with IL-13 and performed transwell migration assay. Figure 6a showed that IL-13 treatment alone enhanced the migration ability of LM2 cells, to a level equivalent to PNR overexpression. Treatment of IL-13 on PNR overexpressed cells only had slight additive effects, probably due to the saturation of migrated cells in this system (Figure 6a). Next, we measured ERK1/2 phosphorylation to indicate the downstream activation of IL-13Rα2. The results showed that IL-13 could stimulate ERK1/2 phosphorylation (Supplementary Figure S3A) and this effect was mediated by IL-13Rα2 because the increase of ERK1/2 phosphorylation was abrogated by IL-13Rα2 knockdown (Figure 6b). U0126, the specific MEK1/2 inhibitor similarly abolished the migration of both control and PNR-overexpressing LM2 cells (Figure 6c). Correspondingly, knockdown of IL-13Rα2 decreased (Figure 6b), whereas overexpression of PNR increased phosphorylation of ERK1/2 level (Figure 6). These results collectively suggest that both IL-13 and PNR induced the migration of LM2 in an IL-13Rα2-dependent manner and that ERK activation was downstream of PNR and IL-13Rα2-mediated migration of breast cancer cells.

PNR directly regulates IL-13Rα2 gene expression

As a nuclear receptor, PNR regulates gene expression by directly binding to the promoter region of target genes including cyclin D1 and TBX2 in Y79 retinoblastoma cell line[19] and ERα in MCF7 and T47D breast cancer cell lines, respectively.[4] As IL-13Rα2 mRNA is increased by PNR overexpression, we hypothesized that PNR may regulate IL-13Rα2 mRNA stability or regulate IL-13Rα2 gene expression by direct association with the IL-13Rα2 promoter. Our result that PNR overexpression did not affect IL-13Rα2 transcript stability after actinomycin D treatment excluded the first probability (Supplementary Figure S3B). We went on to predict the potential binding sites of PNR on IL-13Rα2 promoter using the MatInspector software (MatInspector, Genomatix, München, Germany).[33] The PNR-binding sites were predicted not at the distal promoter but at the proximal promoter (data not shown). IL-13 had been reported to increase IL-13Rα2 gene expression by regulating the distal promoter.[13] However, the regulation of distal promoter does not seem to be the main mechanism in breast cancer cells because IL-13 treatment alone failed to increase IL-13Rα2 mRNA level in both MDA-MB-231 and LM2 cells (Supplementary Figure S3C). This notion was further supported by the IL-13Rα2 distal promoter-driven luciferase reporter

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

assay where PNR was unable to increase the luciferase activity (data not shown). To examine whether PNR influenced the proximal promoter of IL-13Rα2, we performed the luciferase reporter assay by generating the reporters driven by the terminally deleted IL-13Rα2 promoter constructs in HEK293T cells (Figure 7a). Two regions of IL-13Rα2 promoter were highly regulated in transcriptional activation assay (Figure 7a). The first region from −209 to the transcriptional start site contains an AP-1-binding site. A previous study had shown that AP-1 is a transcriptional factor of IL-13Rα2.[12] The second region from −1200 to −545 contains a putative PNR-binding site shown in Figure 7d as predicted by the MatInspector software and this also agrees with the formerly identified PNR consensus sequence.[1,34] PNR expression could further enhance the transcriptional activation of the full-length promoter reporter construct (−1690 to +229) but not the truncated one (−209 to +229) (Figure 7b). Mutations of PNR in the conserved DNA binding domain were previously observed in enhanced S-cone syndrome[35,36] and affect its homodimerization, interaction with cofactors and DNA binding.[37] Thus, we tested whether PNR mutations would affect the activation of IL-13Rα2 promoter. The two mutations, R76W and G88W in the DNA binding domain abolished the activation (Figure 7c), suggesting that the physical interaction of PNR with IL-13Rα2 promoter is required for IL-13Rα2 activation. In keeping with this finding, mutation of the consensus PNR-binding site on IL-13Rα2 promoter also abolished the activation (Figure 7d).

Further, we designed specific primers encompassing these two regions for chromatin immunoprecipitation (ChIP) assay. The ChIP results showed that PNR was indeed associated with IL-13Rα2 promoter (−834 to −323 bp), which was consistent with the result of luciferase reporter assay, whereas no PNR could be detected at the AP-1-binding site (−249 to +229 bp) (Figure 8a). c-Jun is a member of the AP-1 family.[38] Using α-c-Jun antibody for ChIP, we confirmed c-Jun binding at the AP-1 site but not at the PNR-binding site (Figure 8a). The co-immunoprecipitation experiment revealed no direct interaction between PNR and c-Jun (data not shown). To further validate that IL-13Rα2 is transcriptionally activated by PNR, we examined RNA Pol II binding, the acetyl-histone H3 level and H3K4me3 level, which are indicators of transcriptionally active genes[39] at IL-13Rα2 promoter region. The results showed that acetyl-histone H3 levels are elevated throughout the entire region (P1–P3) and Pol II binding and H3K4me3 level were elevated by overexpressing PNR at PNR-binding site, which coincide with the activation of IL-13Rα2 gene (Figures 8b and d). Taken together, our results indicate that PNR regulates transcription of IL-13Rα2 by direct association with the promoter. Figure 9 summarizes our findings that PNR directly regulates IL-13Rα2 gene expression to enhance the IL-13Rα2-mediated downstream ERK activation. The IL-13/IL--13Rα2-mediated signal transduction has critical roles in migration and metastasis of ERα-negative cells. The IL-13 stimulation also has an important role in breast cancer cell migration and metastasis based on our *in vitro* functional assays, which is supported by the positive correlation between high IL-13 mRNA level with the poor overall survival of breast cancer (Supplementary Figure S4).

DISCUSSION

Emerging evidence suggest that PNR has a role in breast cancer, in particular in ERα-positive breast cancer. In ERα-positive cells, PNR activates ERα target gene expression and

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

increases the proliferation rate of MCF7 and T47D cells. We found that in ERα-negative cells, PNR activates IL-13Rα2 gene expression (Figures 4 and 5) that leads to the increase of migration and metastasis of LM2 cells (Figures 2 and 3). Interestingly, PNR does not regulate the expression of IL-13Rα1, nor a positive correlation could be detected between the expression of PNR and IL-13Rα1 in primary human breast cancer (Supplementary Figure S2). These pieces of evidence suggest that PNR regulation of IL-13 signaling is specified through the membrane-bound IL-13Rα2 that activates downstream ERK1/2 signaling cascades to promote metastasis. Thus inhibiting PNR activity would represent a significant means to inhibit metastasis in breast cancer cells.

Existing literatures reported controversial roles of IL-13Rα2 in breast cancer. One study showed that overexpression of IL-13Rα2 inhibits growth of human breast and pancreatic tumors in the immunodefficient mice but has no effect on cell growth *in vitro.*[40] The anti-tumorigenic activity of IL-13Rα2 may not recapitulate the bona fide function of IL-13Rα2 because of overexpression and variability derived from single clones. In another study, Dr Massagué's group demonstrated that IL-13Rα2 did not affect the primary tumor outgrowth but served as a biomarker and potent mediator of breast cancer lung metastasis.[15,23,24,41] In concordance with previous study that IL-13Rα2 behaves as a signaling receptor rather than a decoy receptor to intercept IL-13,[7] our study underscores the significance of IL-13/ IL-13Rα2-mediated signaling in potentiating breast cancer cell migration (Figure 6) and the PNR-induced gene activation (Figures 4 and 7). It is believed that the expression level of IL-13Rα2 affects the receptor distribution and IL-13 signaling.[42] Interestingly, despite LM2 was derived from MDA-MB-231 cells, PNR only increased migration of LM2 cells but not of MDA-MB-231 cells (Figure 2). The difference could be attributed to activation of additional metastasis-related genes other than IL-13Rα2 in LM2 cells but not in MDA-MB-231 cells (Figure 4). This result also implicated that IL-13Rα2 expression level alone was insufficient to determine the invasiveness potential of MDA-MB-231 cells. Notably, we found that IL-13Rα2 and IL-13Rα1 were reversely expressed in LM2 and MDA-MB-231 cells (Supplementary Figure S5). Although IL-13Rα2 was much highly expressed in LM2 cells as compared with MDA-MB-231 cell, IL-13Rα1 showed the opposite trend. Hence, the two IL-13 receptors may form a negative regulatory loop and the ratio of IL-13Rα2 to IL-13Rα1 may be indicative of metastasis potential of cancer cells. A previous study[15] showed that the lung metastasis frequency of a subclone (4175) of LM2 cells in the nude mice was 7/13 when injecting into the mammary fat pad and surgically removed the tumors when the size reached 300 mm^3. Our results showed a much lower incidence of lung metastasis. The discrepancy may be due to the difference in experimental procedures and cell population. In our experiment, the tumors were allowed to grow but not surgically removed. Moreover, the LM2 cell line we used was a pool of different clones. Although some studies used tail vein injection as a model to monitor lung metastasis, we chose to use orthotropic xenograft model because this model better recapitulates the multi-step metastasis. Importantly, we could observe significant difference with regards to the tendency for lung metastasis of LM2 cells overexpressing PNR vs the control group.

PNR does not affect cell proliferation *in vitro* (Figure 1d), while promoting tumor growth *in vivo* (Figure 3). A key difference between these two systems is the tumor microenvironment

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

that may display features of Th2 inflammation,[43] which promotes tumor development. Studies have shown that thymic stromal lymphopoietin, an inflammatory cytokine either expressed by breast cancer cells or cancer-associated fibroblasts of prostate cancer, lead to the production of IL-13 by Th2 cells to promote tumor growth.[43–45] Although multiple lines of evidence have implicated the role of IL-13 signaling in breast cancer development, the mechanism by which IL-13 promotes breast tumor growth remains elusive. Here we showed that IL-13 stimulates tumor growth and migration via IL-13Rα2 and the effect was potentiated by PNR. Interestingly, overexpression of PNR strongly enhances thymic stromal lymphopoietin expression upon stimulation by PMA and ionomycin (Supplementary Figure S6), suggesting that PNR may be the central regulator of IL-13-IL-13Rα2 signaling *in vivo* via regulating both IL-13 secretion and IL-13Rα2 expression. This model could explain that IL-13 regulates breast tumor growth by activating IL-13Rα2 and this effect could be amplified by PNR. The regulation of thymic stromal lymphopoietin by PNR adds another layer of complexity, indicating that the communication between cancer cells and immune cells in the tumor microenvironment may have important roles in tumor metastasis *in vivo*.

Signal transducer and activator of transcription 6 and AP-1 complex were known to regulate IL-13Rα2 transcription by binding to the distal and proximal promoters on IL-13Rα2[12,13] in keratinocytes and glioma cells, respectively. However, how IL-13Rα2 is transcriptionally regulated in breast cancer remains unknown. Our study suggests that PNR and c-Jun may have independent or synergistic effects on IL-13Rα2 regulation. PNR could either activate or repress gene expression.[46] In this study, PNR acts as a transcriptional activator probably by binding to the unidentified physiological agonists, leading to the recruitment of cofactors such as NRL, CRX, Reverbα and PIAS3.[3,4,16,18,47] The recently resolved crystal structure of PNR supports that PNR could accommodate 13-cis retinoic acid as an agonist.[48] The molecular mechanism by which PNR regulates IL-13Rα2 gene expression warrants further investigations.

It is well known that nuclear receptor and epithelial cell growth factor could have functional crosstalk that has clinical implication in breast cancer.[49] We have demonstrated that PNR, an orphan nuclear receptor, transcriptionally activates IL-13Rα2, and further transduces downstream signals via the mitogen-activated protein kinase pathways (Figure 6), leading to breast cancer migration and metastasis. The cell-based observation needs to be validated in human tumor and serum samples. Secreted IL-13 protein levels in serum should also be measured to provide basis for evaluation whether IL-13 signaling contributes to the crosstalk between tumor cells and microenvironment. Although the physiological ligands of PNR have not been identified, numerous efforts have been made to identify small molecule agonist of PNR for treatment of retinal-related disease.[50–53] As PNR activates ERα in ERα-positive breast cancer cells[4] and regulates IL-13Rα2 in ERα-negative cells to promote tumor metastasis, we believe that small molecule inhibitors of PNR may exert clinical benefit to breast cancer patients. The crystal structure of PNR ligand-binding domain has recently been resolved,[48] which provides structural basis for *in silico* screening of small molecules that either activate PNR for the treatment of retinal-related diseases or inhibit PNR for the treatment of breast cancer. Given that IL-13Rα2 is overexpressed in various solid tumor tissues and recently shown to be a novel tumor antigen that could be targeted for

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

immunotherapy,[14] a recombinant fusion IL-13 cytotoxin termed IL-13-PE38QQR has been developed and elicited potent anti-tumor activity in various human tumor graft murine models and several phase I/II clinical trials[14,54,55] to treat patients with malignant brain tumors.[14] This cytotoxin, however, has not yet applied to human breast cancer. On the basis of our finding that PNR regulates tumor metastasis via activation of IL-13Rα2 pathway, modulating the activity of PNR in conjunction with inhibiting the IL-13Rα2-mediated signaling pathway may elicit synergistic therapeutic effects on breast cancer, in particular on ERα-negative breast cancers whose current treatment is limited to chemotherapy.

MATERIALS AND METHODS

Cell culture

LM2 cell line[15] is a kind gift from Dr Joan Massagué. All of the other cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). HEK293T, MCF7, MDA-MB-231 and LM2 breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco) at 37° with 5% CO_2. T47D cell line and BT549 cell line were maintained in Dulbecco's modified Eagle's medium/F12 (Gibco) and RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, respectively. Cell counting was performed as previously described.[56] For PNR knockdown, 50 nM negative control or PNR small interfering RNA (Invitrogen, Carlsbad, CA, USA) was transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) for 72 h before harvesting the cells.

Virus packaging, infection and stable cell line generation

Retroviruses and lentiviruses were packaged as previously described.[51,56] Cells were selected with 800 μg/ml G418 (RPI, Mount Prospect, IL, USA) for PNR overexpression or 2 μg/ml puromycin (RPI) for IL-13Rα2 knockdown for a week to generate stable cell lines.

Immunofluorescence

Immunofluorescence was performed as previously described.[56]

Western blot analysis

Western blot analysis was performed as previously described.[51] Anti-PNR (Genemed Synthesis, San Antonio, TX, USA),[51] IL-13Rα2 (R&D Systems, Minneapolis, MN, USA), FLAG (Sigma, St Louis, MO, USA), ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), phosphor-ERK1/2 (Cell Signaling Technology) and Hsp90 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were used for western Blot.

Wound-healing migration assay

Wound-healing assay was performed as previously described.[57]

Transwell migration assay

Transwell assay was performed as previously described.[56] For IL-13 stimulation experiment, cells were pre-treated with IL-13 (20 ng/ml) (Cell Signaling Technology) for 10

h. For the ERK1/2 inhibition experiment, cells were pre-treated with U0126 mitogen-activated protein kinase inhibitor (10 μM) (Tocris Bioscience, Ellisville, MO, USA) for 1 h before plating onto the transwell inserts.

Two-dimensional colony formation assay

Colony formation assay was performed as previously described.[58]

Adhesion assay

The adhesion assay was performed as previously described.[59]

Animal study

All animal work was performed in accordance with protocols approved by the Animal Care and Use Committee of the University of Wisconsin-Madison, Madison, WI, USA. LM2 cells were infected with retroviruses expressing GFP or PNR. In all, 1×10^6 cells were injected bilaterally into the inguinal mammary fat pads of 5- to 6-week-old ovariectomized athymic nude-Foxn1nu mice ($n = 5$ per group). Tumor size was determined using caliper measurement and the tumor volume was calculated using the formula volume = $\pi/6 \times L \times W2$.

Immunohistochemistry

Lung tissues were subjected to hematoxylin and eosin staining and immunohistochemistry as previously described.[60] Staining for tumor and lung tissues was performed on paraffin-embedded sections. In all, 1 μg/ml blocking peptides for anti-PNR antibody (GeneMed Synthesis) were pre-incubated with anti-PNR antibody for 1 h. The sequences of the blocking peptides are: CLSQHSKAHHPSQP and CPETRGLKDPEH-VEALQD. Rabbit anti-PNR (GeneMed Synthesis), mouse anti-rabbit human Ki67 (Biocare Medical, Concord, CA, USA) and anti-mouse firefly luciferase (Invitrogen) primary antibodies were used.

Quantitative real-time–PCR

Quantitative real-time–PCR was performed as previously described.[51] Primers sequences (IDT, Coralville, IA, USA) used in this study were listed in Supplementary Table S1.

ChIP assay

ChIP assay was performed as previously described.[56] The sequences of the primers used for PCR are listed in Supplementary Table S1. Anti-FLAG (Sigma), c-Jun (Santa Cruz Biotechnology), acetyl-histone H3 (Millipore, Billerica, MA, USA), Pol II (Millipore) and H3K4me3 (Diagenode, Denville, NJ, USA) antibodies were used in ChIP assays.

Luciferase reporter assay

Luciferase assays were performed as previously described.[51]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Statistical analysis

All the results are representative of at least three independent experiments. Statistical significance was calculated using a two-sided Student *t*-test. *P<0.05; **P<0.01; ***P<0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by DOD ERA of HOPE Scholar Award (W81XWYH-11–1–0237) to WX.

References

1. Kobayashi M, Takezawa S, Hara K, Yu RT, Umesono Y, Agata K, et al. Identification of a photoreceptor cell-specific nuclear receptor. Proc Natl Acad Sci USA. 1999; 96:4814–4819. [PubMed: 10220376]
2. Bumsted O'Brien KM, Cheng H, Jiang Y, Schulte D, Swaroop A, Hendrickson AE. Expression of photoreceptor-specific nuclear receptor NR2E3 in rod photo-receptors of fetal human retina. Invest Ophthalmol Vis Sci. 2004; 45:2807–2812. [PubMed: 15277507]
3. Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, Saunders TL, et al. Nrl is required for rod photoreceptor development. Nat Genet. 2001; 29:447–452. [PubMed: 11694879]
4. Park YY, Kim K, Kim SB, Hennessy BT, Kim SM, Park ES, et al. Reconstruction of nuclear receptor network reveals that NR2E3 is a novel upstream regulator of ESR1 in breast cancer. EMBO Mol Med. 2012; 4:52–67. [PubMed: 22174013]
5. Muscat GE, Eriksson NA, Byth K, Loi S, Graham D, Jindal S, et al. Research resource: nuclear receptors as transcriptome: discriminant and prognostic value in breast cancer. Mol Endocrinol. 2013; 27:350–365. [PubMed: 23292282]
6. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, et al. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature. 1993; 362:248–250. [PubMed: 8096327]
7. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med. 2006; 12:99–106. [PubMed: 16327802]
8. Hershey GK. IL-13 receptors and signaling pathways: an evolving web. J Allergy Clin Immunol. 2003; 111:677–690. quiz 691. [PubMed: 12704343]
9. Barderas R, Bartolome RA, Fernandez-Acenero MJ, Torres S, Casal JI. High expression of IL-13 receptor alpha2 in colorectal cancer is associated with invasion, liver metastasis, and poor prognosis. Cancer Res. 2012; 72:2780–2790. [PubMed: 22505647]
10. Fujisawa T, Joshi B, Nakajima A, Puri RK. A novel role of interleukin-13 receptor alpha2 in pancreatic cancer invasion and metastasis. Cancer Res. 2009; 69:8678–8685. [PubMed: 19887609]
11. Fujisawa T, Joshi BH, Puri RK. IL-13 regulates cancer invasion and metastasis through IL-13Ralpha2 via ERK/AP-1 pathway in mouse model of human ovarian cancer. Int J Cancer. 2012; 131:344–356. [PubMed: 21858811]
12. Wu AH, Low WC. Molecular cloning and identification of the human interleukin 13 alpha 2 receptor (IL-13Ra2) promoter. Neuro Oncol. 2003; 5:179–187. [PubMed: 12816724]
13. David MD, Bertoglio J, Pierre J. Functional characterization of IL-13 receptor alpha2 gene promoter: a critical role of the transcription factor STAT6 for regulated expression. Oncogene. 2003; 22:3386–3394. [PubMed: 12776189]
14. Joshi BH, Puri RK. IL-13 receptor-alpha2: a novel target for cancer therapy. Immunotherapy. 2009; 1:321–327. [PubMed: 20635949]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

15. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. Nature. 2005; 436:518–524. [PubMed: 16049480]
16. Cheng H, Khanna H, Oh EC, Hicks D, Mitton KP, Swaroop A. Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photo-receptors. Hum Mol Genet. 2004; 13:1563–1575. [PubMed: 15190009]
17. Mollema NJ, Yuan Y, Jelcick AS, Sachs AJ, von Alpen D, Schorderet D, et al. Nuclear receptor Reverb alpha (Nr1d1) functions in concert with Nr2e3 to regulate transcriptional networks in the retina. PloS one. 2011; 6:e17494. [PubMed: 21408158]
18. Peng GH, Ahmad O, Ahmad F, Liu J, Chen S. The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet. 2005; 14:747–764. [PubMed: 15689355]
19. Takezawa S, Yokoyama A, Okada M, Fujiki R, Iriyama A, Yanagi Y, et al. A cell cycle-dependent co-repressor mediates photoreceptor cell-specific nuclear receptor function. EMBO J. 2007; 26:764–774. [PubMed: 17255935]
20. Gupta GP, Massague J. Cancer metastasis: building a framework. Cell. 2006; 127:679–695. [PubMed: 17110329]
21. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science. 2011; 331:1559–1564. [PubMed: 21436443]
22. Steeg PS. Metastasis suppressors alter the signal transduction of cancer cells. Nat Rev Cancer. 2003; 3:55–63. [PubMed: 12509767]
23. Christofori G. Cancer: division of labour. Nature. 2007; 446:735–736. [PubMed: 17429382]
24. Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, et al. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature. 2007; 446:765–770. [PubMed: 17429393]
25. Jezequel P, Frenel JS, Campion L, Guerin-Charbonnel C, Gouraud W, Ricolleau G, et al. bc-GenExMiner 3.0: new mining module computes breast cancer gene expression correlation analyses. Database (Oxford). 2013; 2013:bas060. [PubMed: 23325629]
26. Obiri NI, Leland P, Murata T, Debinski W, Puri RK. The IL-13 receptor structure differs on various cell types and may share more than one component with IL-4 receptor. J Immunol. 1997; 158:756–764. [PubMed: 8992992]
27. Murata T, Obiri NI, Debinski W, Puri RK. Structure of IL-13 receptor: analysis of subunit composition in cancer and immune cells. Biochem Biophys Res Commun. 1997; 238:90–94. [PubMed: 9299458]
28. Aman MJ, Tayebi N, Obiri NI, Puri RK, Modi WS, Leonard WJ. cDNA cloning and characterization of the human interleukin 13 receptor alpha chain. J Biol Chem. 1996; 271:29265–29270. [PubMed: 8910586]
29. Hilton DJ, Zhang JG, Metcalf D, Alexander WS, Nicola NA, Willson TA. Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. Proc Natl Acad Sci USA. 1996; 93:497–501. [PubMed: 8552669]
30. Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, et al. Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. FEBS Lett. 1997; 401:163–166. [PubMed: 9013879]
31. Caput D, Laurent P, Kaghad M, Lelias JM, Lefort S, Vita N, et al. Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-5 receptor alpha chain. J Biol Chem. 1996; 271:16921–16926. [PubMed: 8663118]
32. Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL, et al. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. J Immunol. 1998; 161:2317–2324. [PubMed: 9725226]
33. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, et al. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics. 2005; 21:2933–2942. [PubMed: 15860560]
34. Pankratz MJ, Busch M, Hoch M, Seifert E, Jackle H. Spatial control of the gap gene knirps in the *Drosophila* embryo by posterior morphogen system. Science. 1992; 255:986–989. [PubMed: 1546296]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

35. Sharon D, Sandberg MA, Caruso RC, Berson EL, Dryja TP. Shared mutations in NR2E3 in enhanced S-cone syndrome, Goldmann-Favre syndrome, and many cases of clumped pigmentary retinal degeneration. Arch Ophthalmol. 2003; 121:1316–1323. [PubMed: 12963616]
36. Haider NB, Jacobson SG, Cideciyan AV, Swiderski R, Streb LM, Searby C, et al. Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. Nat Genet. 2000; 24:127–131. [PubMed: 10655056]
37. Roduit R, Escher P, Schorderet DF. Mutations in the DNA-binding domain of NR2E3 affect in vivo dimerization and interaction with CRX. PloS one. 2009; 4:e7379. [PubMed: 19823680]
38. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem. 1995; 270:16483–16486. [PubMed: 7622446]
39. Yan C, Boyd DD. Histone H3 acetylation and H3 K4 methylation define distinct chromatin regions permissive for transgene expression. Mol Cell Biol. 2006; 26:6357–6371. [PubMed: 16914722]
40. Kawakami K, Kawakami M, Snoy PJ, Husain SR, Puri RK. In vivo overexpression of IL-13 receptor alpha2 chain inhibits tumorigenicity of human breast and pancreatic tumors in immunodefficient mice. J Exp Med. 2001; 194:1743–1754. [PubMed: 11748276]
41. Ring BZ, Ross DT. Predicting the sites of metastases. Genome Biol. 2005; 6:241. [PubMed: 16356275]
42. Daines MO, Tabata Y, Walker BA, Chen W, Warrier MR, Basu S, et al. Level of expression of IL-13R alpha 2 impacts receptor distribution and IL-13 signaling. J Immunol. 2006; 176:7495–7501. [PubMed: 16751396]
43. Pedroza-Gonzalez A, Xu K, Wu TC, Aspord C, Tindle S, Marches F, et al. Thymic stromal lymphopoietin fosters human breast tumor growth by promoting type 2 inflammation. J Exp Med. 2011; 208:479–490. [PubMed: 21339324]
44. De Monte L, Reni M, Tassi E, Clavenna D, Papa I, Recalde H, et al. Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. J Exp Med. 2011; 208:469–478. [PubMed: 21339327]
45. Olkhanud PB, Rochman Y, Bodogai M, Malchinkhuu E, Wejksza K, Xu M, et al. Thymic stromal lymphopoietin is a key mediator of breast cancer progression. J Immunol. 2011; 186:5656–5662. [PubMed: 21490155]
46. Webber AL, Hodor P, Thut CJ, Vogt TF, Zhang T, Holder DJ, et al. Dual role of Nr2e3 in photoreceptor development and maintenance. Exp Eye Res. 2008; 87:35–48. [PubMed: 18547563]
47. Oh EC, Cheng H, Hao H, Jia L, Khan NW, Swaroop A. Rod differentiation factor NRL activates the expression of nuclear receptor NR2E3 to suppress the development of cone photoreceptors. Brain Res. 2008; 1236:16–29. [PubMed: 18294621]
48. Tan MH, Zhou XE, Soon FF, Li X, Li J, Yong EL, et al. The crystal structure of the orphan nuclear receptor NR2E3/PNR ligand binding domain reveals a dimeric auto-repressed conformation. PloS One. 2013; 8:e74359. [PubMed: 24069298]
49. Conzen SD. Minireview: nuclear receptors and breast cancer. Mol Endocrinol. 2008; 22:2215–2228. [PubMed: 18417735]
50. Wolkenberg SE, Zhao Z, Kapitskaya M, Webber AL, Petrukhin K, Tang YS, et al. Identification of potent agonists of photoreceptor-specific nuclear receptor (NR2E3) and preparation of a radioligand. Bioorg Med Chem Lett. 2006; 16:5001–5004. [PubMed: 16879962]
51. Zhao Z, Wang L, Wen Z, Ayaz-Guner S, Wang Y, Ahlquist P, et al. Systematic analyses of the cytotoxic effects of compound 11a, a putative synthetic agonist of photoreceptor-specific nuclear receptor (PNR), in cancer cell lines. PloS One. 2013; 8:e75198. [PubMed: 24066170]
52. Kapitskaya M, Cunningham ME, Lacson R, Kornienko O, Bednar B, Petrukhin K. Development of the high throughput screening assay for identification of agonists of an orphan nuclear receptor. Assay Drug Dev Technol. 2006; 4:253–262. [PubMed: 16834531]
53. Qin Q, Knapinska A, Dobri N, Madoux F, Chase P, Hodder P, et al. In pursuit of synthetic modulators for the orphan retina-specific nuclear receptor NR2E3. J Ocul Pharmacol Ther. 2013; 29:298–309. [PubMed: 23098562]
54. Husain SR, Puri RK. Interleukin-13 receptor-directed cytotoxin for malignant glioma therapy: from bench to bedside. J Neurooncol. 2003; 65:37–48. [PubMed: 14649884]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

55. Puri RK, Leland P, Obiri NI, Husain SR, Kreitman RJ, Haas GP, et al. Targeting of interleukin-13 receptor on human renal cell carcinoma cells by a recombinant chimeric protein composed of interleukin-13 and a truncated form of pseudomonas exotoxin A (PE38QQR). Blood. 1996; 87:4333–4339. [PubMed: 8639793]

56. Wang L, Zhao Z, Meyer MB, Saha S, Yu M, Guo A, et al. CARM1 methylates chromatin remodeling factor BAF155 to enhance tumor progression and metastasis. Cancer cell. 2014; 25:21–36. [PubMed: 24434208]

57. Powell E, Shanle E, Brinkman A, Li J, Keles S, Wisinski KB, et al. Identification of estrogen receptor dimer selective ligands reveals growth-inhibitory effects on cells that co-express ERalpha and ERbeta. PloS One. 2012; 7:e30993. [PubMed: 22347418]

58. Noetzel E, Rose M, Bornemann J, Gajewski M, Knuchel R, Dahl E. Nuclear transport receptor karyopherin-alpha2 promotes malignant breast cancer phenotypes in vitro. Oncogene. 2012; 31:2101–2114. [PubMed: 21909132]

59. Wang L, Shen Y, Song R, Sun Y, Xu J, Xu Q. An anticancer effect of curcumin mediated by down-regulating phosphatase of regenerating liver-3 expression on highly metastatic melanoma cells. Mol Pharmacol. 2009; 76:1238–1245. [PubMed: 19779032]

60. Wang L, Charoensuksai P, Watson NJ, Wang X, Zhao Z, Coriano CG, et al. CARM1 automethylation is controlled at the level of alternative splicing. Nucleic Acids Res. 2013; 41:6870–6880. [PubMed: 23723242]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

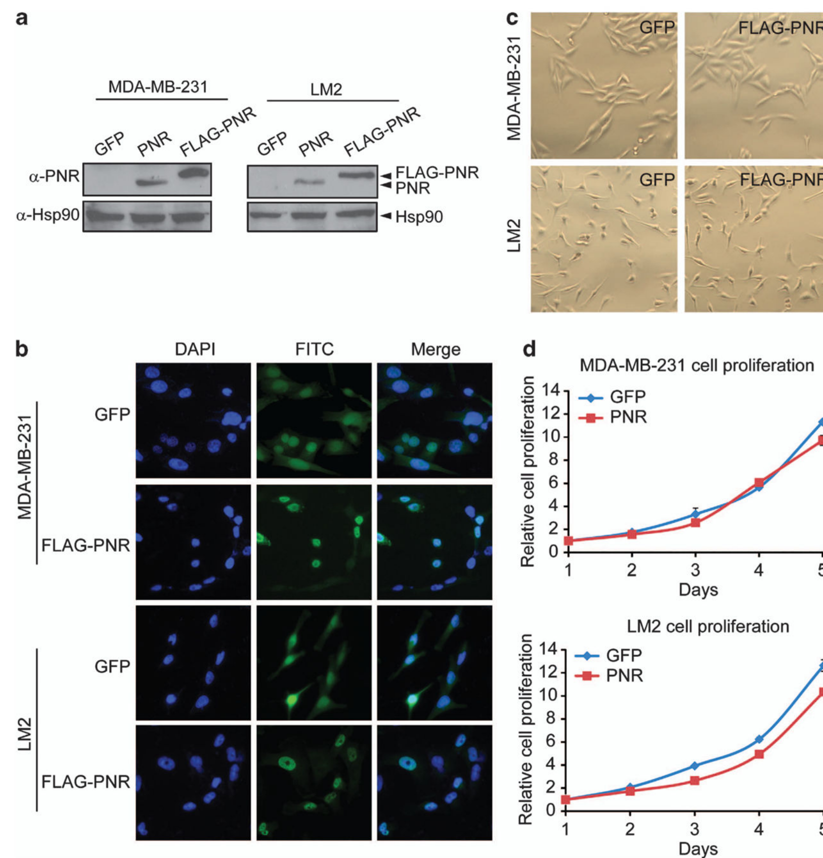

Figure 1.

Generation of PNR-overexpressing MDA-MB-231 and LM2 cells. MDA-MB-231 and LM2 cells were infected with retroviruses expressing GFP, PNR or FLAG-PNR. (**a**) Western blot analysis of PNR expression using anti-PNR antibody. Hsp90 was used as loading control. (**b**) FLAG-PNR protein is localized to nucleus in MDA-MB-231 and LM2 cells by immunofluorescence using anti-FLAG antibody. Objective magnification, × 20. (**c**) PNR overexpression did not alter cell morphology as detected under the bright field. (**d**) PNR overexpression did not affect the cell proliferation rate of MDA-MB-231 and its derivative clone LM2. The error bars represent ±s.d. values of three independent measurements.

Author Manuscript

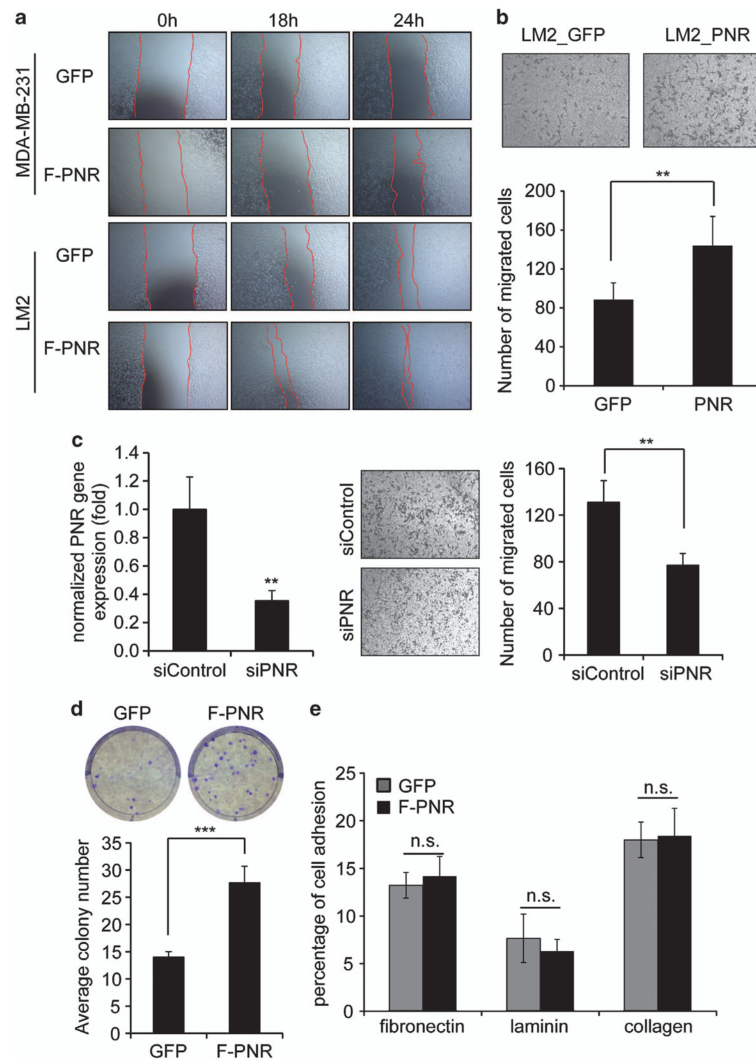

Figure 2.

PNR overexpression increased migration and colony formation of LM2 cells. MDA-MB-231 and LM2 cells were infected with retroviruses expressing GFP, PNR or FLAG-PNR. (**a**) Cells were subjected to the wound-healing assay and representative photos were taken at × 10 magnification immediately after the scratch and after 18 and 24 h. PNR was either overexpressed (**b**) or knocked down (**c**) in LM2 cells and subjected to transwell migration assay. After fixation and staining, images were captured at × 10 magnification. Quantification was the average of five independent fields. (**d**) Two-dimensional colony formation assay of 100 cells for 2 weeks. (**e**) Cell adhesion assay performed on plates coated with fibronectin, laminin, collagen-I or bovine serum albumin (BSA) as negative control for normalization. The error bars represent ±s.d. values of three independent measurements. $^{**}P<0.01$; $^{***}P<0.001$; NS, not significant.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

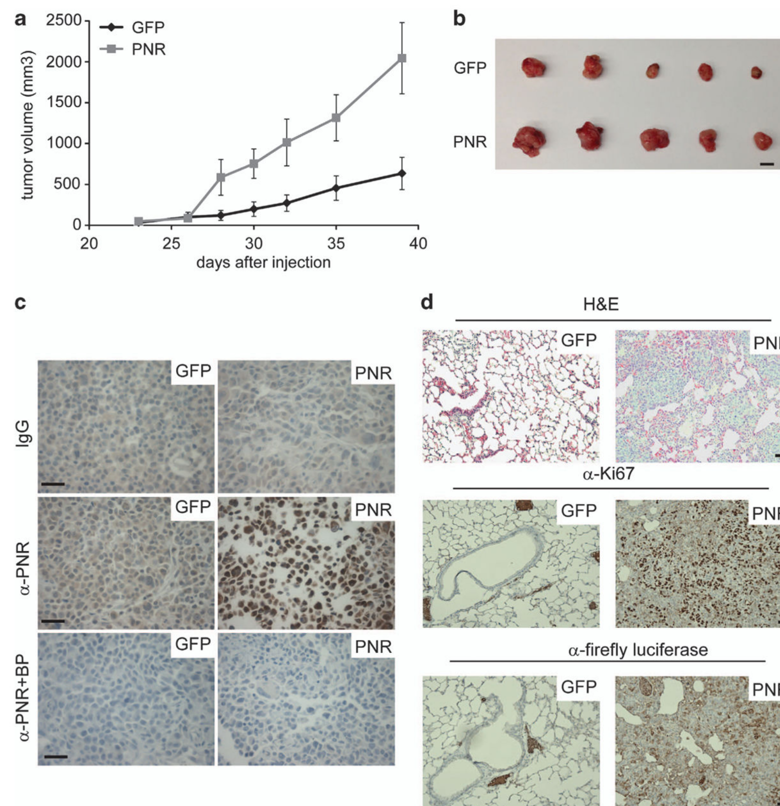

Figure 3.

Overexpressing of PNR promotes primary tumor growth and lung metastasis in xenograft mouse models. (**a**) Overexpressing of PNR increases the growth rate of primary tumors and tumor volume. GFP or PNR-overexpressing LM2 cells were bilaterally injected into the inguinal mammary fat pads. Data are the mean ±s.e.m. of five mice in each group. (**b**) Representative photograph of tumors from GFP or PNR-overexpressing LM2 xenograft. Scale bar, 1 cm. (**c**) Immunohistochemistry (IHC) staining of PNR in GFP or PNR-overexpressing tumor grafts. Immunoglobulin G (IgG) serves as the negative control for PNR IHC. Blocking peptides (1 μg/ml) were used to assess the specificity of the anti-PNR antibody. (**d**) The lung tissues were stained with hematoxylin and eosin, human Ki67 and firefly luciferase. Scale bar, 50 μm.

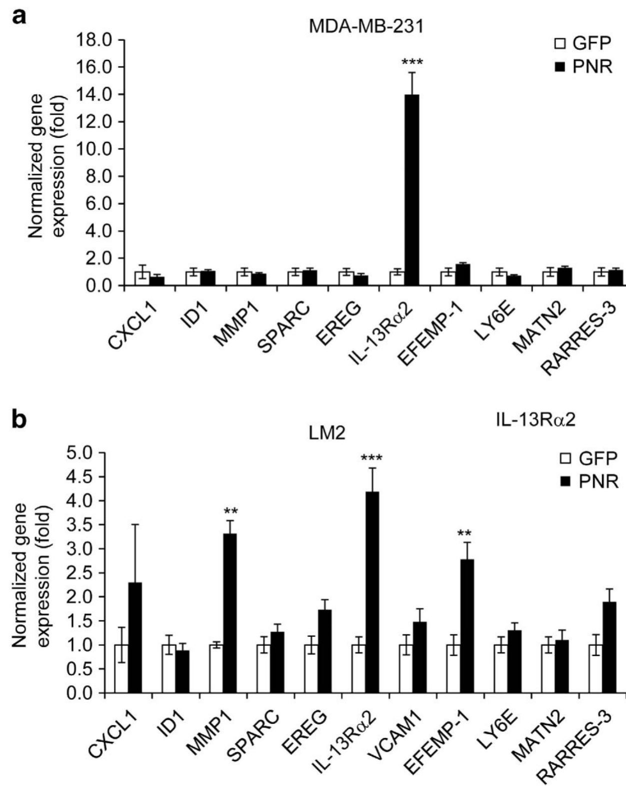

Figure 4.

Overexpressing of PNR increased IL-13Rα2 mRNA level. Total RNA from GFP or PNR overexpressed MDA-MB-231 (**a**) and LM2 (**b**) cells were collected for quantitative real-time (qRT)–PCR to examine the relative expression of metastasis-related gene changes. The error bars represent ±s.d. values. $^{**}P<0.01$; $^{***}P<0.001$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

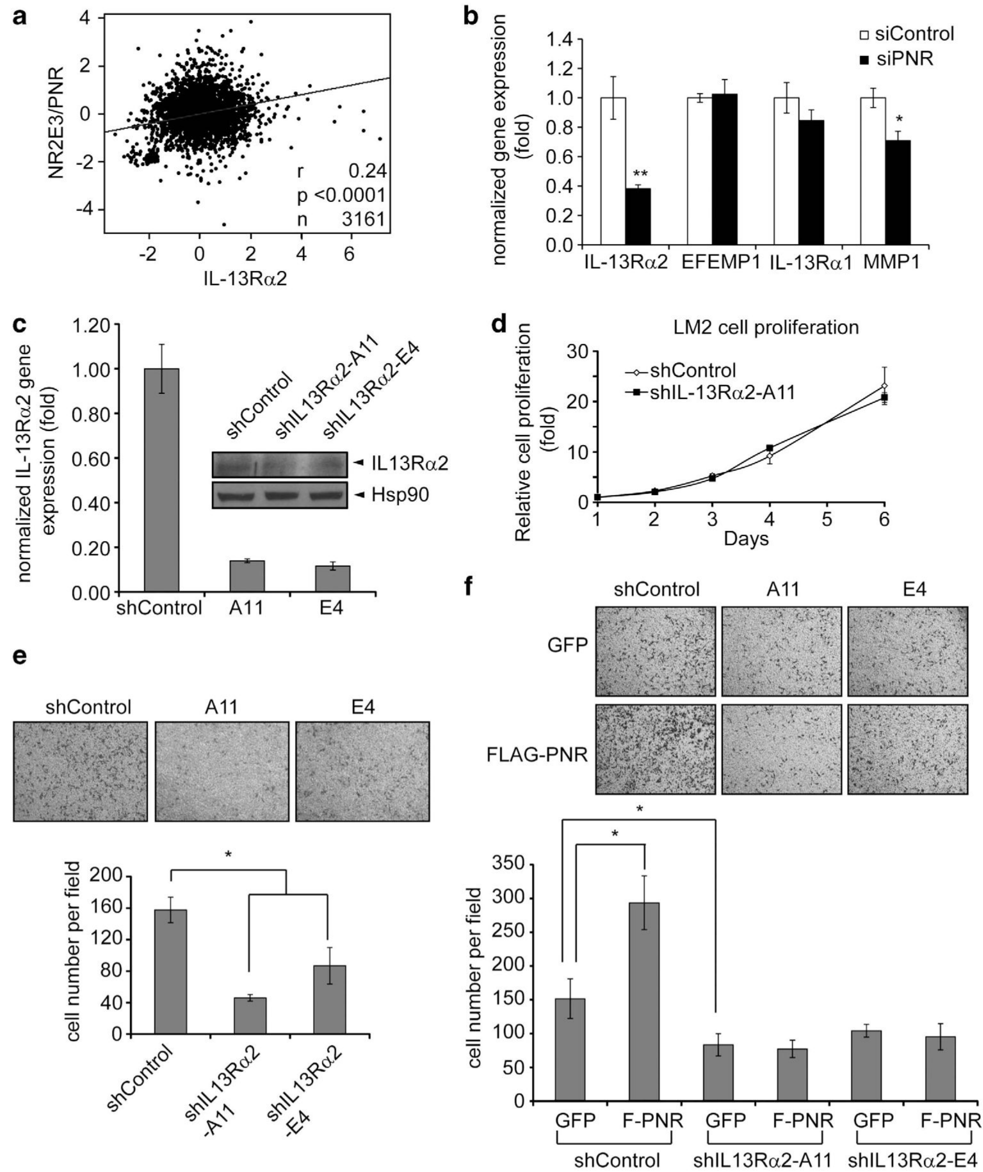

Figure 5.

The PNR-induced cell migration is dependent on IL-13Rα2. (**a**) The Pearson's pairwise correlation plot depicts the positive correlation of PNR and IL-13Rα2 mRNA expression in human breast tumor datasets collected in bc-GenExMiner Database. (**b**) The mRNA levels of the indicated genes were examined by quantitative real-time (qRT)–PCR after 72 h of PNR knockdown by siRNA. (**c**) Two short hairpin RNA (shRNAs), A11 and E4, could effectively knockdown IL-13Rα2 in LM2 cells as determined at RNA and protein levels using quantitative–PCR and western blot, respectively. (**d**) The proliferation of LM2 cells was not affected by IL-13Rα2 knockdown. (**e**) Knockdown of IL-13Rα2 in LM2 cells decreased cell migration shown by the transwell migration assay. Representative images of the migrated cells in lower chamber (top) and the quantification of migrated cells (bottom) are shown. (**f**) PNR-induced migration increase depends on IL-13Rα2. PNR was

overexpressed in the control or IL-13Rα2 knocked down LM2 cells followed by transwell migration assay. *$P<0.05$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

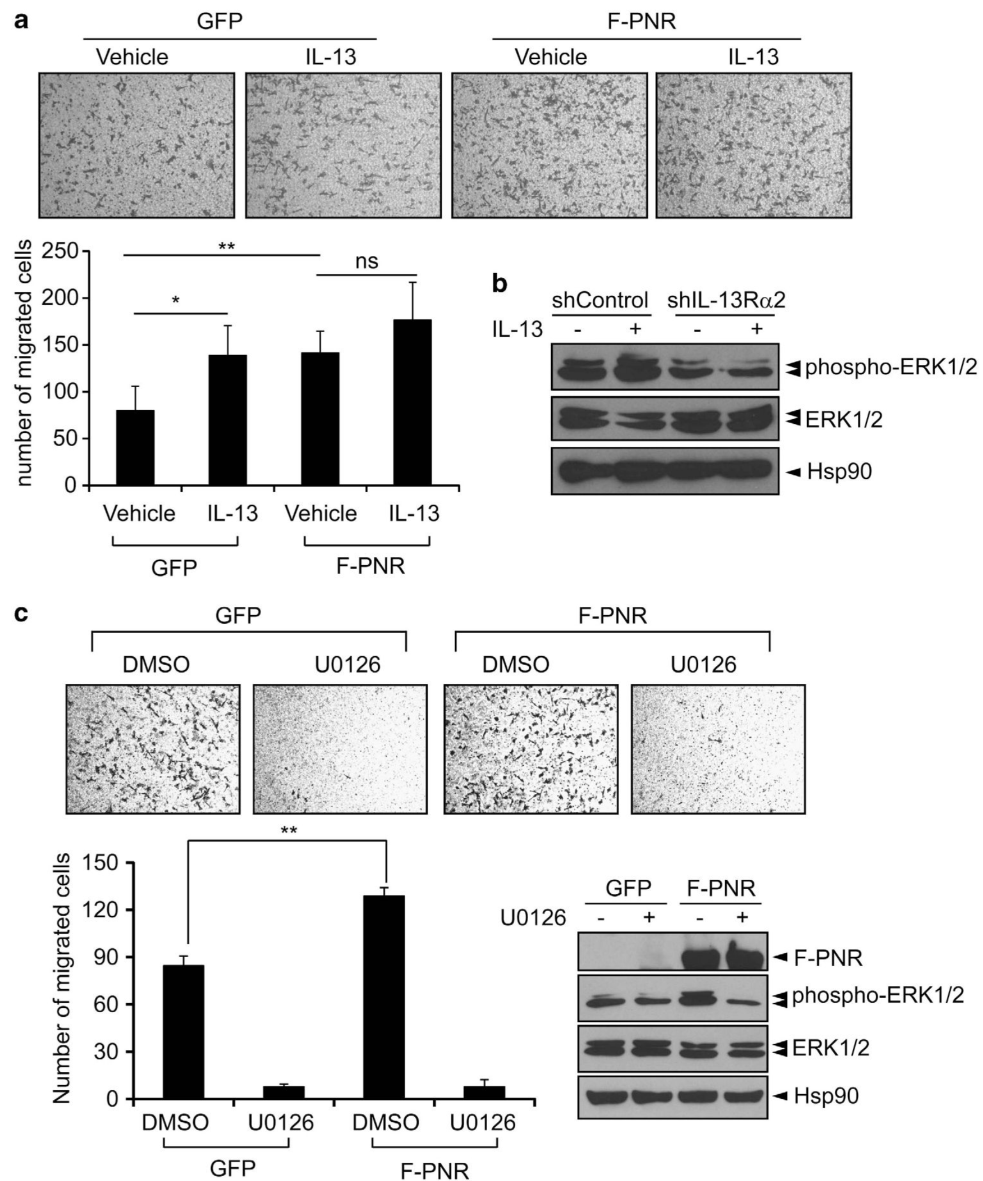

Figure 6.

ERK phosphorylation and cell migration stimulated by IL-13 depend on IL-13Rα2. (**a**) LM2 cells overexpressing GFP control or F-PNR were pre-treated with IL-13 (20 ng/ml) for 10 h in Dulbecco's modified Eagle's medium (DMEM) plus 0.5% fetal bovine serum (FBS) and then subjected to the transwell migration assay. (**b**) LM2 cells were treated with IL-13 (20 ng/ml) in the normal media for 2 h. Cell lysates were harvested for western blot to detect phospho-ERK1/2 and total ERK1/2 levels. Hsp90 was used as loading control. (**c**) Mitogen-activated protein kinase (MAPK) inhibitor U0126 blocks cell migration and ERK1/2 phosphorylation in LM2 cells overexpressing F-PNR. GFP or F-PNR-overexpressing cells were pre-treated with U0126 (10 μM) for 1 h in DMEM plus 0.5% FBS and then subjected to the transwell migration assay. PNR, phospho-ERK1/2 and total ERK1/2 in cell lysates were detected by western blot with Hsp90 as loading control. *$P<0.05$; **$P<0.01$.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

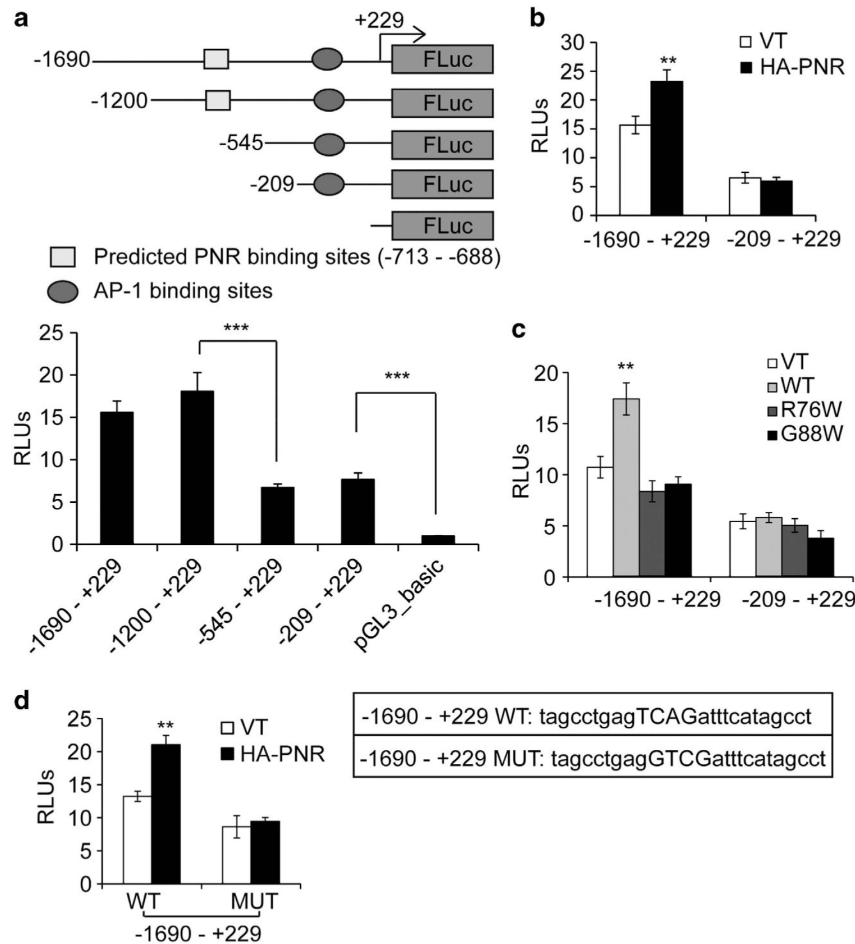

Figure 7.

PNR activates transcription of IL-13Rα2 in the luciferase reporter assays. (**a**) The truncated constructs of IL-13Rα2 promoter were transfected into HEK293T cells for luciferase reporter assay. (**b**) Vector control (VT) or HA-PNR was co-transfected with IL-13Rα2 promoters (−1690 to +299 or −209 to +229) for luciferase reporter assay. (**c**) Wild-type PNR but not DNA-binding mutants activates IL-13Rα2 promoter in reporter assay. (**d**) Mutation of the consensus PNR-binding site in IL-13Rα2 promoter results in abrogation of IL-13Rα2 in the reporter assay. $^{**}P<0.01$; $^{***}P<0.001$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

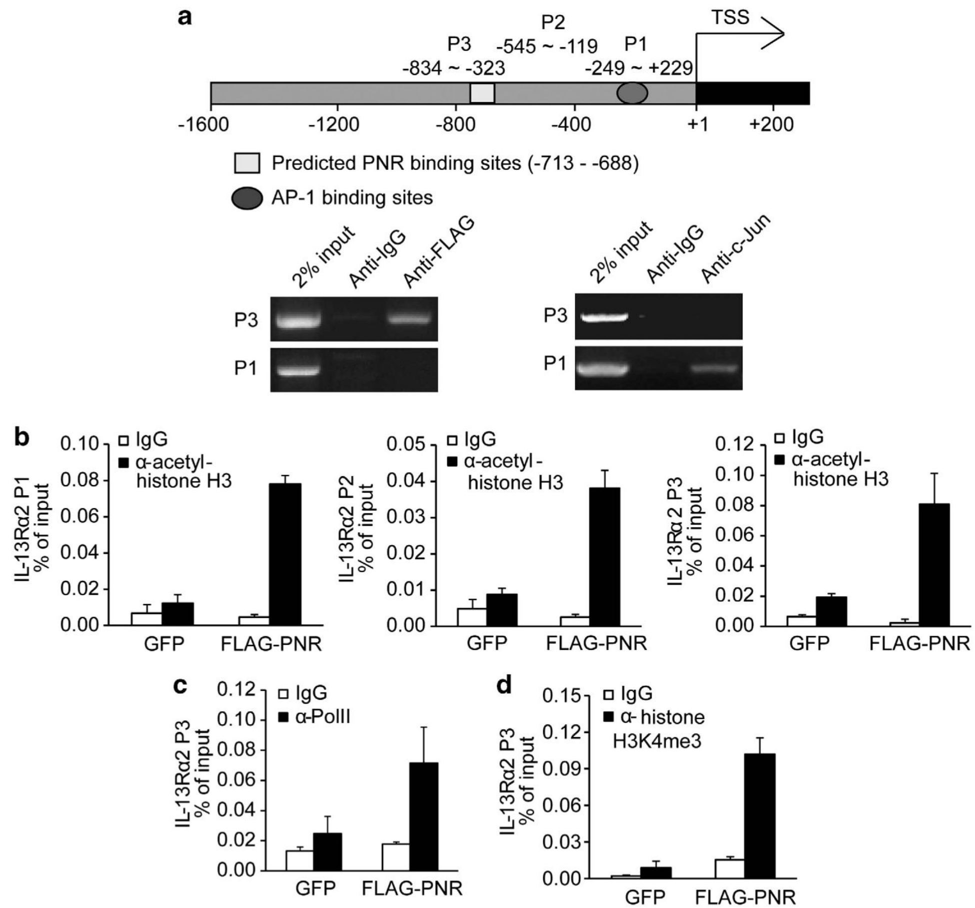

Figure 8.

PNR activates the transcription of IL-13Rα2 via direct association with IL-13Rα2 promoter. (**a**) PNR and c-Jun were detected at discrete sites on the IL-13Rα2 promoter by ChIP assay in MDA-MB-231 cells overexpressing FLAG-PNR. ChIP assays were performed with anti-FLAG or anti-c-Jun antibodies with anti-IgG as a negative control. PCR amplified products were shown on agarose gel (bottom). Increased acetylation of histone H3 (**b**) in the entire transcription factor-binding region (P1–P3), Pol II binding (**c**) and H3K4me3 (**d**) to P3 were detected on the IL-13Rα2 promoter by ChIP assay using indicated antibodies. P1, P2 and P3 correspond to (−249 to +229), (−545 to −119) and (−834 to −232) on IL-13Rα2 promoter, respectively.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

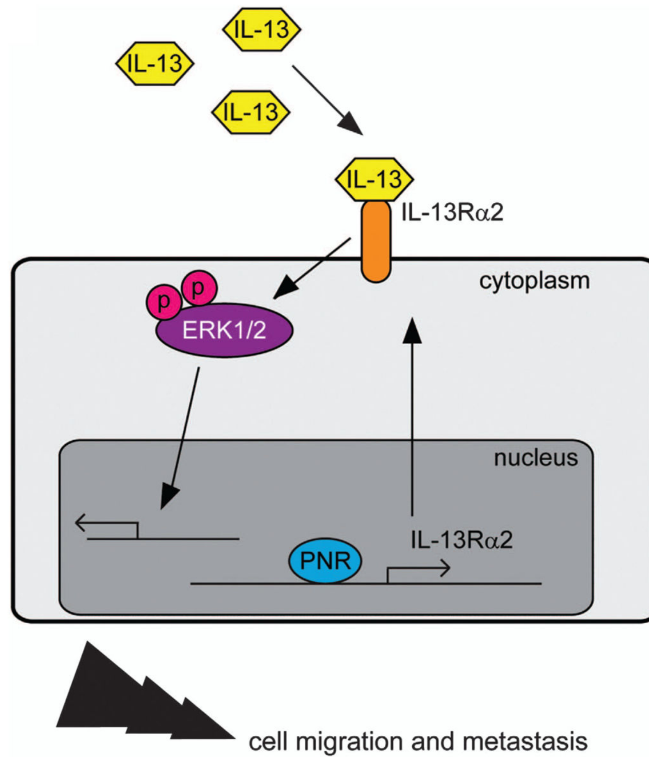

Figure 9.

The proposed mechanism of PNR-induced, IL-13Rα2-mediated breast cancer cell migration and metastasis. PNR directly binds to the promoter region of IL-13Rα2 to activate its transcription. The increase of IL-13Rα2 protein level in conjunction with IL-13 treatment activate downstream mitogen-activated protein kinase (MAPK)-ERK pathway, which leads to breast cancer cell migration and metastasis.