Oncogene. Author manuscript; available in PMC 2010 May 19.

Published in final edited form as:

Oncogene. 2009 November 19; 28(46): 4041–4052. doi:10.1038/onc.2009.259.

The *NRG1* gene is frequently silenced by methylation in breast cancers and is a strong candidate for the 8p tumour suppressor gene

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Abstract

Neuregulin-1 (NRG1) is both a candidate oncogene and candidate tumour suppressor gene. It encodes the heregulins and other mitogenic ligands for the *ERBB* family, but it also causes apoptosis in *NRG1*-expressing cells. We found that most breast cancer cell lines had reduced or undetectable expression of *NRG1*. This included cell lines that had translocation breaks in the gene. Similarly, expression in cancers was generally comparable to or less than various normal breast samples. Many non-expressing cell lines had extensive methylation of the CpG island at the principal transcription start site at exon 2 of *NRG1*. Expression was reactivated by demethylation. Many tumours also showed methylation, while normal mammary epithelial fragments had none. Lower *NRG1* expression correlated with higher methylation. siRNA-mediated depletion of *NRG1* increased net proliferation, in a normal breast cell line and a breast cancer cell line that expressed *NRG1*. The short arm of chromosome 8 is frequently lost in epithelial cancers, and *NRG1* is the most centromeric gene that is always affected. *NRG1* may therefore be the major tumour suppressor gene postulated to be on 8p: it is in the correct location, is anti-proliferative, and is silenced in many breast cancers.

Keywords

breast cancer; NRG1; heregulins; DNA methylation; tumour suppressor gene; chromosome rearrangement

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Conflict of Interest The authors declare no conflict of interest.

Supplementary information is available at Oncogene's website.

Introduction

The *NRG1* (neuregulin-1) gene has been proposed both as a candidate oncogene and a candidate tumour suppressor gene. It seems likely to play a role in epithelial cancers, since it encodes ligands that bind to the ERBB/HER/EGFR family of receptors. These ligands, originally known as the heregulins alpha and beta, neu differentiation factor/NDF, SMDF and glial growth factor II, are made by alternative splicing, and include forms that are transmembrane, externally membrane-bound, shed, secreted or intracellular (Falls, 2003; Hayes & Gullick, 2008). They bind to ERBB3 or ERBB4, which probably signal as heterodimers with ERBB2 (HER2).

Although the *NRG1*-encoded proteins are usually thought of as mitogens, they can also be powerfully pro-apoptotic: in particular, expressing *NRG1* in cells can cause apoptosis of the expressing cell (Weinstein et al., 1998).

The *NRG1* gene has been identified as a potential cancer-critical gene in two, apparently contradictory, contexts. Firstly, it is the prime candidate for the major tumour suppressor gene thought to be on 8p, the short arm of chromosome 8. Loss of 8p is one of the most frequent genomic events in epithelial cancers, including breast, colon, bladder and prostate. This has been shown successively by loss of heterozygosity (LOH), comparative genomic hybridization (CGH) and array-CGH studies (for references see Birnbaum et al., 2003; Pole et al., 2006). The classical interpretation of this loss of 8p would be that there is a tumour suppressor gene there. We previously mapped the 8p losses in carcinoma cell lines by fluorescence-in situ hybridization (FISH) and array-comparative genomic hybridization (array-CGH) and found that almost all breaks were proximal to, or actually within *NRG1*, making *NRG1* and genes immediately telomeric to *NRG1* the prime candidates for such a tumour suppressor (Pole et al., 2006; Cooke et al., 2008).

Secondly, *NRG1* could be an oncogene because it appears to be the target of chromosome translocations in breast cancer. In the breast cancer cell line MDAMB-175, a translocation has fused the 3′ end of *NRG1*, including the receptor-binding domain, downstream of *ODZ4/DOC4*, creating a secreted protein with biological activity (Schaefer et al., 1997; Wang et al., 1999; Liu et al., 1999). We and others showed that there are breakpoints within *NRG1* in a number of other breast cancer cell lines and in around 6% of breast tumours, all preserving the 3′ end of the gene (Adélaïde et al., 2003; Huang et al., 2004; Prentice et al., 2005). One interpretation of this is that *NRG1* is activated by fusion or promoter insertion. However, other explanations have been suggested (see Discussion; Weinstein & Leder, 2000; Birnbaum et al., 2003; Prentice et al., 2005).

We set out to investigate the role of *NRG1* in breast cancer, beginning by measuring expression of *NRG1* in normal breast and breast cancers.

Results

NRG1 transcription start sites and exon usage in breast

To quantitate expression of *NRG1*, which has many alternative splice forms (Figure 1a; Falls, 2003), preliminary RT-PCR experiments (not shown) were carried out, to find which of the three main transcription start sites were used in the cell lines, and whether there were major variations in exon usage. These experiments showed that, generally, where a cell line expressed any *NRG1*, all exons tested were expressed, except exon 1. This implied that the exon 1 transcription start site was not used in these samples and transcription was from the start sites in exon 2 and exon 7 (Figure 1a). Also, the pattern of exon usage was rather similar across the samples. This allowed us to broadly quantitate *NRG1* expression using a

single PCR primer pair that spanned exons 4 to 6, which are in almost all transcripts initiated in exon 2.

One exception requires comment. In MDA-MB-175, expression of all exons except exons 1 and 2 was detected. This line expresses a fusion of *NRG1* that splices in at exon 3. Although the fusion cDNA that was originally described lacked the transmembrane and cytoplasmic domains (Schaefer et al., 1997), we detected all the 3′ exons that we tested for, so the fusion gene expresses alternative isoforms including the intracellular domains, as also found by Adélaïde et al. (2003).

Expression of *NRG1* is low in breast cancers

Quantitative PCR using the primer pair spanning exons 4 to 6 showed that many breast cancer cell lines expressed little or no *NRG1* (Figure 1b), while normal mammary epithelium, both as cell lines (Figure 1) and cells (see below), did express *NRG1*. Similar results were obtained with a primer pair in exon 8, which encodes the receptor-binding domain and is included in almost all isoforms, but is short and a poor target for PCR (Supplementary Figure 1a).

Western blotting was consistent with the mRNA expression data: a single major band of ~75 kDa was seen in the normal breast cell line HB4a and cancer lines that showed substantial mRNA expression (Supplementary Figure 1b), while no band was detected in T-47D, a representative line that expressed no mRNA. 75 KDa is the expected size for intact heregulins (see e.g. Deadwyler et al., 2000), all of which are expressed from exon 2.

A similar pattern was seen when *NRG1* mRNA expression was measured in breast tumour samples, and cells and tissue from normal breast (Figure 2a). The highest expression was seen in purified myoepithelial cells and normal breast epithelial ‘organoids’, i.e. intact, uncultured epithelial fragments made up of both myoepithelial and luminal epithelial cells, isolated by collagenase digestion of breast tissue (Edwards et al., 1986). Purified luminal cells and commercial RNA from long-term primary cultures expressed less. Among the tumours, three of six samples of purified tumour cells from pleural or ascitic effusions showed essentially no expression. Tumour tissue samples showed a range of expression, mostly equal to or less than the normal samples, with 25% of the 58 samples expressing less than any of the normal samples. Some of this expression may well be from stromal cells, which are typically 30% or more of the cells present, so it is possible that many of the tumours have lost expression of *NRG1*, as for the cell lines.

The CpG Island in *NRG1* is methylated in breast cancer cell lines

We therefore investigated whether *NRG1* expression might be silenced by DNA methylation. Bisulphite sequencing analysis of the CpG island around the exon 2 transcription start site (Figure 1a) showed that it was heavily methylated in 10 of 19 (53%) breast cancer cell lines tested, while the remaining lines and the non-cancer breast cell lines HB4A and HMT-3552 showed little methylation (Figure 1c).

Methylation correlated closely with an absence of *NRG1* transcripts (Figure 1b, c). The ten breast cancer cell lines with methylation showed no expression, while the lines that have *NRG1* transcripts had low DNA methylation. A further small group of breast cancer cell lines, MDA-MB-157, UACC 812, HCC 1187 and SUM52, lacked *NRG1* transcripts and had low DNA methylation at the CpG island, suggesting that *NRG1* can also be inactivated by mechanisms other than DNA methylation.

Azacytidine treatment

Two cell lines that had heavy CpG island methylation, HCC 1500 and MDA-MB-361, were treated with the demethylating agent 5-aza-2′-deoxycytidine. Treatment activated transcription of *NRG1* (Supplementary Figure 2).

NRG1 is methylated in tumours but not in normal breast tissue

To quantitate methylation in a panel of tissue samples, we used pyrosequencing of bisulphite-treated DNA (Yang et al., 2004). A 29-bp sequence was selected from the CpG island (Figure 1a) that included six CpGs that were always methylated in breast cancer cell lines that showed methylation, but were unmethylated in non-cancer breast lines (Figure 1c).

No DNA methylation at *NRG1* was detected in uncultured, normal breast epithelium (Figure 2b), obtained in the form of ‘organoids’.

In contrast, many tumours showed substantial DNA methylation of the 29-bp sequence, half the 59 tumour tissue samples averaging 24% or more methylation (Figure 2b). Average methylation ranged from 0 to 60%, the upper limit of 60% presumably reflecting the presence of normal cells in the samples. To attempt to address this, purified tumour cells from six pleural or ascitic effusions were also analysed (Figure 2b). One pleural effusion sample showed substantial methylation, averaging about 40% over the pyrosequenced CpGs.

When methylation of this six-CpG sequence was compared with expression of *NRG1*, there was a trend to decrease in *NRG1* expression with increase in DNA methylation (Figure 2c). More precisely, Figure 2c suggests that, as for the cell lines, some tumours have *NRG1* expression reduced by methylation, while others have *NRG1* silenced by other mechanisms. The latter group would be those with low expression but little or no methylation (circled in Figure 2c). If these cases were set aside, the correlation between lower expression and increasing methylation was clear and highly significant.

Since *NRG1* may be a tumour suppressor that is inactivated by a two-hit mechanism, with one hit often being loss of distal 8p, it was interesting to divide the tumours according to whether they had lost 8p, using existing array-CGH data (Chin et al., 2007). There was no profound difference in expression and methylation between the tumours with and without 8p loss, but the 18 tumours with 8p loss showed a tighter correlation between expression and methylation, provided the outlier group were excluded (Figure 2c).

Knockdown of *NRG1* can increase net cell proliferation

These observations suggested that down-regulating *NRG1* expression in mammary epithelial cells might give cells a survival advantage. We reduced *NRG1* expression by stable expression of siRNA constructs in the normal breast luminal epithelial cell line HB4a and in HCC1806, a breast cancer cell line that expresses a relatively high level of *NRG1*, comparable to HB4a (Figure 1b). Multiple siRNA constructs were designed, giving three independent siRNA treatments for HB4a and two treatments for HCC1806.

Net proliferation was modestly but consistently increased, independent of the siRNA construct used, even though only a modest reduction of expression was seen (Figure 3). This result together with the methylation data suggests that *NRG1* acts as a tumour suppressor.

Expression of receptors for *NRG1*

To aid interpretation of our *NRG1* data, we also measured expression of *ERBB2, ERBB3* and *ERBB4* in our cell line panel (Supplementary Figure 3). There was a strong negative

association between *NRG1* expression and high *ERBB*-family expression (Figure 4): the few breast cell lines with near-normal or raised *NRG1* expression had low or negligible expression of *ERBB2, ERBB3* and *ERBB4*, while the lines that showed high expression of any *ERBB* gene expressed little or no *NRG1*. The lines that did express some *NRG1* together with raised *ERBB2, ERBB3* and/or *ERBB4* were HCC1806, MDA-MB-175, HCC1937 and ZR-75-1, all of which have breaks in *NRG1*, and CAMA-1, for which the genomic structure of *NRG1* is not known.

Discussion

Our findings suggest that *NRG1* is frequently inactivated in breast cancer and behaves as a tumour suppressor. *NRG1* was expressed in normal human breast, by both luminal and myoepithelial cells, while in many breast cancer cell lines there was little or no expression. Absence of expression was often associated with DNA methylation of the CpG island at the principal transcription start site for *NRG1* in cell lines, and most tumours also showed DNA methylation. Reducing *NRG1* expression in cell lines increased net cell proliferation. Others have shown that expressing *NRG1* in a breast cancer cell line by transfection causes apoptosis (Weinstein et al., 1998).

NRG1 in normal breast

We found that both luminal and basal/myoepithelial cells in non-pregnant human breast epithelium express *NRG1*. Previous data on *NRG1* expression in normal mammary gland do not give a clear picture. In mouse, Yang et al. (1995) reported that the NRG1 isoform heregulin-alpha was expressed by the stroma in late pregnancy, implying that there was little expression in the epithelium, particularly in non-pregnant epithelium. However, Schaefer et al. (1997) reported expression from normal human breast tissue (and absence in several breast cancer cell lines), and Aguilar et al. (1999) and Adélaïde et al. (2003) reported expression of *NRG1* mRNA in cultured normal human mammary epithelial cells, which are likely to be predominantly basal/myoepithelial cells (Clarke et al., 2005).

In vivo experiments on the role of *NRG1* in mouse mammary gland seem to have neglected the resting mammary gland (Britsch, 2007). Attention has been focussed on heregulin alpha's critical role in late pregnancy and lactation, already mentioned (Yang et al., 1995). This is supported by several findings: the activated rat *ERBB2, neu*^{T}, when introduced into mammary epithelium caused development of clusters of alveolar-like structures (Bradbury et al., 1993); heregulin implants in the gland stimulated duct branching (Jones et al., 1996), and a knockout mouse with a stop codon in exon 9 of *NRG1*, preventing translation of alpha isoforms, had retarded lactational development (Li et al., 2002).

One proposed role for *NRG1* in epithelia in general, consistent with it being implicated in cancer development, is as a mediator of wound-healing (Vermeer et al., 2003). At least in human airway epithelium, NRG1 protein is produced at the apical face of the polarised epithelium while its receptor(s) are basolateral, so that NRG1 signalling would indicate breaching of the epithelium (Vermeer et al., 2003). Histology shows that malignant epithelium is often, perhaps always, defective in the ability to restore a single, intact epithelial surface, implying a defect in wound-healing.

The significance of translocation breakpoints in *NRG1*

One postulated role for *NRG1* was as an oncogene activated by chromosome translocation in breast cancers. Our data do not in general support this. As noted in the Introduction, about 6% of breast tumours have translocations or other genome rearrangements with breakpoints within *NRG1*, retaining the 3′ end of the gene (Huang et al., 2004), and in one cell line,

MDA-MB-175, the translocation produces a fusion protein ODZ4-NRG1. This raised the possibility that *NRG1* expression was activated in breast tumours by translocation. However, seven of the cell lines used here have a translocation breakpoint within *NRG1* (Table 1), and they did not show increased expression: two had equivalent expression to the normals, two had substantially less, and three had no detectable expression (Table 1). The one cell line in our whole set that showed slightly increased expression compared to normal epithelium, MDA-MB-415, is not known to have a rearrangement of the NRG1 region, and no rearrangement was detected by FISH with BAC probes (not shown) or by array CGH (Pole et al., 2006). Similarly in the tumours, there was no population with dramatically increased expression.

The breakpoints in NRG1 may therefore in most cases *in*activate one copy of the gene. The gene is extremely large, so the prevalence of breaks within the gene is not particularly surprising (Birnbaum et al., 2003; Prentice et al., 2005).

There may of course be cases where translocation creates an abnormal, or abnormally-regulated *NRG1* product. To date the MDA-MB-175 cell line is the only case with a translocation that is known to result in a fusion gene. It is not known whether the fusion product has modified activity. Weinstein & Leder (2000) suggested that it might not have the pro-apoptotic activities of wild-type NRG1, since the original cDNA clone of the fusion transcript lacked the cytoplasmic, pro-apoptotic exons. However, we and Adélaïde et al. (2003) detected expression of the cytoplasmic exons, so this specific mechanism seems unlikely.

Methylation of the *NRG1* CpG island in breast cancers

Our data suggests that many breast tumours have silenced *NRG1* by aberrant methylation of the CpG island. Many breast cell lines had large numbers of methylated CpGs in the CpG island at the major transcription start of *NRG1*. Partially removing methylation in two cell lines that expressed no *NRG1* restored some expression. Breast tumours, because they contain 30% or more normal cells, give less clear data, but half showed 24% or more methylation averaged over 6 CpGs. No methylation was detected in purified, uncultured epithelium from normal breast, so no substantial population of breast epithelial cells is normally DNA-methylated at *NRG1*. This is consistent with a genome-wide survey of CpG island methylation in 13 normal tissues (including several epithelia but not mammary gland), which found little methylation of this CpG island except light methylation in peripheral blood cells (Rakyan et al., 2008).

An alternative interpretation might be that *NRG1* is silenced by normal differentiation-specific methylation (e.g. Takizawa et al., 2001; Ching et al., 2005), in a small population of epithelial cells in mammary gland that gives rise to tumours. Mammary epithelium comprises several cell types: the outer basal/myoepithelial cells and the inner luminal cells, and there are subpopulations of luminal cell (Kalirai & Clarke, 2006). However, this would not explain why some of the breast tumour cell samples showed low *NRG1* expression without any methylation. We prefer the interpretation that methylation is abnormal and is one of various ways of silencing the gene.

Breast cancer cell lines have been tentatively classified by gene expression into the subsets recognised for breast cancers: luminal-like, basal-like, etc. (Charafe-Jauffret et al, 2006; Neve et al, 2006). Most of our lines are luminal-like, but there was no obvious relationship between this classification and *NRG1* expression, breakpoints within *NRG1*, or methylation (Table 1).

NRG1 has antiproliferative activity

Although proteins encoded by *NRG1* such as the heregulins are thought of as mitogenic, they can also be strongly pro-apoptotic, particularly to the cell that expresses the gene. Leder and coworkers showed that forced expression of *NRG1* causes apoptosis in various cell lines, including MCF7, a breast cancer cell line that does not express *NRG1* (Weinstein et al., 1998). This is a potent activity, since they had discovered it in an unbiased screen for pro-apoptotic cDNAs in HEK293 cells, in which *NRG1* was the only hit (in an incomplete screen) (Grimm & Leder, 1997). Pro-apoptotic activity was independent of ERBB-family receptors and required the C-terminus of *NRG1*, emphasizing that the action was on the expressing cell (Grimm et al., 1998; Weinstein et al., 1998). Exogenous NRG1 proteins can also be anti-proliferative under certain conditions (e.g. Amin et al., 2005; Muraoka-Cook et al., 2006).

Our siRNA experiments suggest a net anti-proliferative effect of *NRG1* expression in our system: down-regulating *NRG1* expression in two cell lines, HB4a and HCC1806, enhanced net cell proliferation. These siRNA results were likely to have been a specific effect, since it is unlikely that off-target effects would increase proliferation, and independent constructs gave similar results.

At least some of this antiproliferative signalling may be extracellular (autocrine or cell-to-cell), since there was a strong negative association between *NRG1* expression and expression of *ERBB2, ERBB3* and *ERBB4* (Figure 4).

NRG1 could be the major tumour suppressor on 8p

The provocative interpretation of our results is that *NRG1* is the long-sought tumour suppressor gene on 8p. Loss of, and/or homozygosity for, distal 8p is one of the most common genomic changes in carcinomas (see Introduction). This suggests that there is a major tumour suppressor gene on 8p, but no convincing candidate has been found (Birnbaum et al., 2003; Cooke et al., 2008). We have previously shown that the breakpoints leading to 8p loss are almost all within or proximal to *NRG1*, consistent with *NRG1*, or a gene immediately telomeric to *NRG1*, being a tumour suppressor gene that drives these losses (Pole et al., 2006; Cooke et al., 2008). The next most telomeric gene, *WRN*, is not a good candidate, although it has been reported to be methylated (Agrelo et al., 2006), since loss of *WRN* compromises telomere replication (Crabbe et al., 2007).

Conclusion

In conclusion, we suggest that NRG1 may be the principal tumour suppressor gene that leads to loss of 8p in many breast and other epithelial cancers. *NRG1* expression seems to be silenced in most breast cancers compared to the main types of mammary epithelial cell—this could be because tumours arise from a specialised population in which *NRG1* is normally silenced, but we prefer the interpretation that *NRG1* is silenced by aberrant methylation or other—as yet unknown—events such as promoter mutation. Expression of *NRG1* in mammary cells is anti-proliferative to the cells that express it. And array-CGH identifies *NRG1* as the gene most likely to be a principal 8p tumour suppressor.

Materials and methods

Cell lines, tumours and normal breast tissue

Cancer cell lines were as described (Pole et al., 2006). The non-cancer lines were from the originators: HB4a is a line immortalised from purified breast luminal epithelial cells

(Stamps et al., 1994) and the HMT3522 line was from fibrocystic (non-cancer) breast (Briand et al., 1987).

The breast tumors were 63 primary operable invasive breast cancers from the Nottingham City Hospital Tumor Bank, which we have extensively profiled (e.g. Garcia et al., 2005; Chin et al., 2007; Naderi et al., 2007). Both cDNA and genomic DNA were available for 54 tumours; a further 4 had cDNA alone, 5 had genomic DNA alone.

Six samples of pure breast tumour cells were from one ascitic and five pleural effusions collected at University College Hospital, London. They were chosen for their high tumour-cell content and treated with red blood cell lysis buffer if heavily contaminated with blood. To purify the tumour cells, macrophages and reactive mesothelial cells were removed by exploiting their rapid adhesion to tissue culture plastic. Cells were incubated in L-15 medium/ 5% FCS for 2 hours at 37°C in large flasks, then the unattached tumour cells aspirated to give estimated >95% tumour cells (MJO'H and RCS, unpublished).

Normal breast from reduction mammoplasty was obtained, with informed consent, from patients aged 18 to 38 years. Epithelial fragments, 'organoids', were prepared by collagenase digestion of tissue without culturing (Edwards et al., 1986). Purified luminal and myoepithelial cells were prepared from primary cultures initiated from organoids that had been trypsinised and fractionated using antibodies and magnetic bead technology (Grigoriadis et al., 2006).

RT-PCR

RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), treated with DNaseI (DNA-free kit, Ambion Division, Applied Biosystems, Foster City, CA) to remove genomic DNA, and was reverse-transcribed using oligo-dT primers and Superscript III (Invitrogen). Real-time RT-PCR for *NRG1* exons 4 to 6 was performed using primers HrgPCRE4F1 (CATTAACAAAGCATCACTGGCT) and hrg3_6R1 (TGAAGAAGTATTTGCTCCTT); primers for exon 8 were HRGE8F1, CTACATCTACATCCACCACTGG and HRGE8R2, TTGCACAAGTATCTCGAGGGGT (chr8:32705009+32705138). SYBR Green PCR Master Mix (Applied Biosystems) was used in an ABI 7900 (Applied Biosystems). GAPDH was used as reference transcript, using the primers GAPDH_1F (GCAAATTCCATGGCACCGT) and GAPDH_1R (TCGCCCCACTTGATTTTGG). Primers for *ERBB2*, *ERBB3* and isoform-specific primers for *ERBB4* are given in Supplementary Figure 3. In preliminary experiments, by conventional RT-PCR, primer pairs were designed within all 17 exons except exons 4-6, 11 and 15.

Western blotting

Monoclonal antibody MAB377 (R&D Systems, Abingdon, UK) was used at 1:200. It had been obtained by immunisation with recombinant human neuregulin1 isoform beta 1 extracellular domain (amino acids 2 - 246, exons 2 to 6, 8 and part of 10), and is expected to detect most isoforms except SMDF and perhaps GGF2. Cell lysates were prepared in the presence of protease inhibitors according to Iyer et al. (2004) and analyzed on 10% polyacrylamide gels. Monoclonal binding was detected with anti-mouse peroxidase conjugate (Dako, California, US) at 1:1000 using the Amersham ECL system (Amersham Biosciences, Uppsala, Sweden). Re-probing for GAPDH used rabbit anti-GAPDH antibody AB9485 (Abcam, Cambridge, UK) at 1:1000.

Bisulphite sequencing

DNA was bisulphite treated using EZ DNA methylation Gold kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. Two overlapping sections of the *NRG1* CpG

island were amplified by PCR from bisulphite-modified DNA. Primers designed for bisulphite-modified DNA (MethPrimer at http://www.urogene.org/methprimer/) were MetHrgF5 (GGGGIAATTGAAAAAGAG) and MeHrgR1 (ACCCACCTAAACTCTAACTACC), located −452 and +106 from the translation start site, and MeHrgF3 (GAGGGATAAATTTTTTTTAAAT) and MeHrgR2 (CTATCCCTTACCCTAAACTCTAAAC), located −94 and +329 from the translation start site (Figure 1a). The PCR products were cloned by TOPO TA Cloning kit (Invitrogen), and 10 clones were sequenced.

For pyrosequencing the target sequence GCGGCGGCGGCTGCCGGACGATGGGAGCG was selected, 32525414 to 32525442 bp on reference sequence NCBI Builds 35 and 36. It was amplified by PCR using MetHrgF5 (used above) and MetHrgF4_Bio primer (5′ biotinylated-ATTTAAAAAAAATTTATCCCTC). The biotinylated PCR product was bound to Streptavidin Sepharose HP (GE Healthcare Amersham, UK), denatured using a 0.2 M NaOH solution, washed with 10mM Tris-Acetate (pH 7.6) and then 70% ethanol using Vacuum Prep Tool (Biotage, Uppsala, Sweden). The purified single-stranded PCR products were released at 80°C in annealing buffer (20mM Tris-Acetate, 2mM Mg-Acetate; pH7.6), mixed with pyrosequencing primer MeHrgSeq_Pur1 (GAGGAGGTTAGGAGTTGA) and sequenced using the PSQ HS 96 Pyrosequencing System and PyroMark MD System (Biotage) with the sequencing reagents Pyro Gold Reagents (Biotage). DNA methylation was quantified using PSQ HS 96A SNP Software and Pyro Q-CpG Software (Biotage). Pyrosequencing agreed with conventional bisulphite sequencing when six representative cell lines were reanalysed, the largest difference in average methylation over the 29-bp fragment being 12%.

Azacytidine treatment

Cell lines were treated with 1 to 5 μM 5-aza-2′-deoxycytidine for 96h (MDA-MB-361) or 76h (HCC1500).

Small interfering RNA (siRNA) knockdown

siRNA constructs were designed to target exons 3 and 4, present in all transcripts that start at the exon 2 CpG island (two constructs); and exons 13, 14 and 17 which are in the cytoplasmic pro-apoptotic region. We could not design siRNAs to uniquely target the ‘universal’ exon, exon 8. Some siRNAs were not very potent in preliminary experiments and these were combined, to give three independent siRNA treatments for HB4a and two treatments for HCC1806. Constructs were generated according to Brummelkamp et al. (2002). Oligos were:

pSuperNRG1_13 (targetted to exon 13)
5′gatccccTCACCATCCTAACCCACCCttcaagagaGGGTGGGTTAGGATGGTGAttttttta and
agcttaaaaaTCACCATCCTAACCCACCCtctcttgaaGGGTGGGTTAGGATGGTGAggg3′

for pSuperNRG1_94 (targetted to exon 14)
5′gatccccCCCATCACTCCACTACTGTttcaagagaACAGTAGTGGAGTGATGGGttttta3′ and
5′agcttaaaaaCCCATCACTCCACTACTGTtctcttgaaACAGTAGTGGAGTGATGGGggg3′;

pSuperNRG1_153 (exon 3)
5′gatccccAAACTAGTCCTTCGGTGTGttcaagagaCACACCGAAGGACTAGTTTttttta3′ and

5′agcttaaaaaAAACTAGTCCTTCGGTGTGtctcttgaaCACACCGAAGGACTAGTTTgg g3′;

pSuperNRG1_380 (exon 4)
5′gatccccCACCATCGTGGAATCAAACttcaagagaGTTTGATTCCACGATGGTGttttta 3′ and
5′agcttaaaaaCACCATCGTGGAATCAAACtctcttgaaGTTTGATTCCACGATGGTGgg g3′;

pSuperNRG1_14 (exon 14)
5′gatccccGAGAAGCAGAGACATCCTTttcaagagaAAGGATGTCTCTGCTTCTCttttta 3′ and
5′agcttaaaaaGAGAAGCAGAGACATCCTTtctcttgaaAAGGATGTCTCTGCTTCTCgg g3′; and

pSuperNRG1_17 (exon 17)
5′gatccccGAAACGACCCAAGACTACGttcaagagaCGTACTCTTGGGTCGTTTCttttta 3′ and
5′agcttaaaaaGAAACGACCCAAGACTACGtctcttgaaCGTACTCTTGGGTCGTTTCgg g3′.

NRG1 target sequences are indicated in capitals, and were designed using OligoEngine Workstation 2 (http://www.oligoengine.com). Annealed oligos were ligated into *Bgl*II and *Hind*III sites of the pSUPER.retro.puro (Brummelkamp et al., 2002) vector and inserts sequenced.

HB4A and HCC 1806 were transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells cultivated on a tissue culture flask (75 cm^2) were incubated with 32μl Lipofectamine together with pSuperNRG1_380 (exon 2) (2μg), a mixture of pSuperNRG1_94 (1μg) and pSuperNRG1_153 (1μg) targetting exons 2 and 14, and a mixture of pSuperNRG1_14 (1μg) and pSuper NRG1_17 (1μg), targetting exons 14 and 17, in 8ml OptiMEM medium (Invitrogen) for 5 hr at 37°C in a CO_2 incubator, then 8ml complete medium with serum was added. Cells were selected in 5μg/ml puromycin (Sigma, Poole, UK) for a month to select stably-transfected cells. Two independent transfections were performed for each combination of constructs. pSUPER.retro.puro was used as a control.

For growth curves, cells were plated in a 6-well tissue culture plate, at two starting densities, and were trypsinized and counted with a Beckman-Coulter ViCell XR Imaging Hemacytometer (Beckman, Fullerton, CA) in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Huai-En Huang for some exploratory expression analysis. Funded principally by the Breast Cancer Campaign and also by Cancer Research UK, Hutchison-Whampoa Ltd. and the Ludwig Institute for Cancer Research.

Work was supported by the Breast Cancer Campaign, Cancer Research UK, Hutchison-Whampoa Ltd. (who endowed the buildings in which the work was done) and the Ludwig Institute for Cancer Research.

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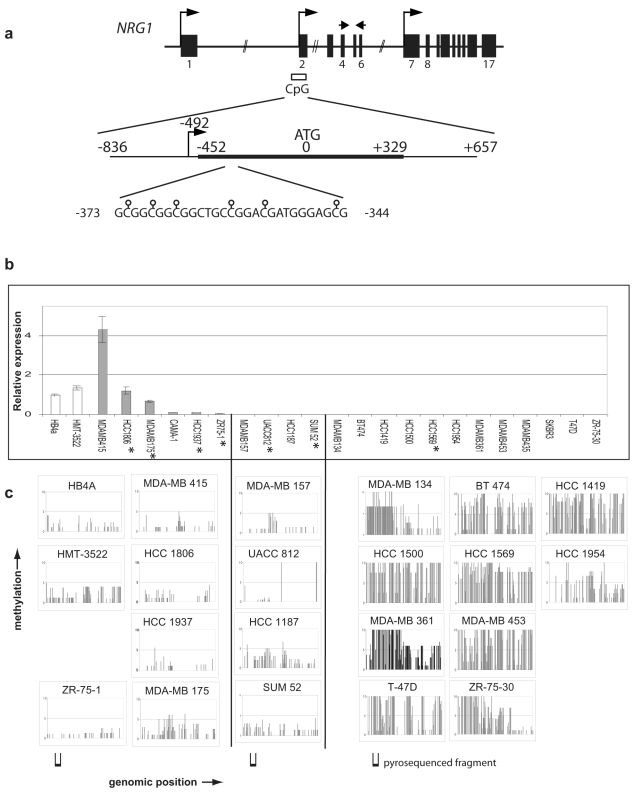

Figure 1.

Expression and CpG methylation of *NRG1* in cell lines.

a Exons of NRG1 and the CpG island. Exons are according to Falls (2003) with introns not to scale. Arrows mark the major transcription start sites at exons 1, 2 and 7 (for additional transcript start sites and exons see Steinthorsdottir et al., (2004). Arrowheads, primer sites in exons 4 and 6 used for quantitative PCR shown in **b**. CpG, CpG island identified at exon 2. Underneath, the CpG island: thin line, whole island, thick line, section analysed for methylation by cloning and sequencing, with positions relative to the translation start site of exon 2 (ATG at 32,525,787 bp on the reference genome sequence Builds 35 and 36). Below this the 29bp sequence selected for pyrosequencing, with the six cytosines of CpGs marked with a circle.

b Expression of NRG1 in cell lines. Top panel, expression by real-time quantitative RT-PCR, using primers shown in **a**, relative to normal breast luminal epithelial cell line, HB4a, on a linear scale. Open bars, lines from non-cancer breast; grey bars, cancer cell lines in order of decreasing expression. Cell lines marked with an asterisk have translocation breaks within NRG1, all of which preserve the 3′ end of the gene.

c Methylation levels over the CpG island (shown in **a**), determined by conventional bisulphite sequencing. In these histograms, vertical lines represent number of methylated copies of individual CpGs in ten clones, plotted against genomic position. The left-hand group of lines have some expression and low methylation; the middle group show little expression and little methylation, the right-hand group show little expression and high methylation. The brackets below the methylation histograms show the region selected for pyrosequencing in Figure 2.

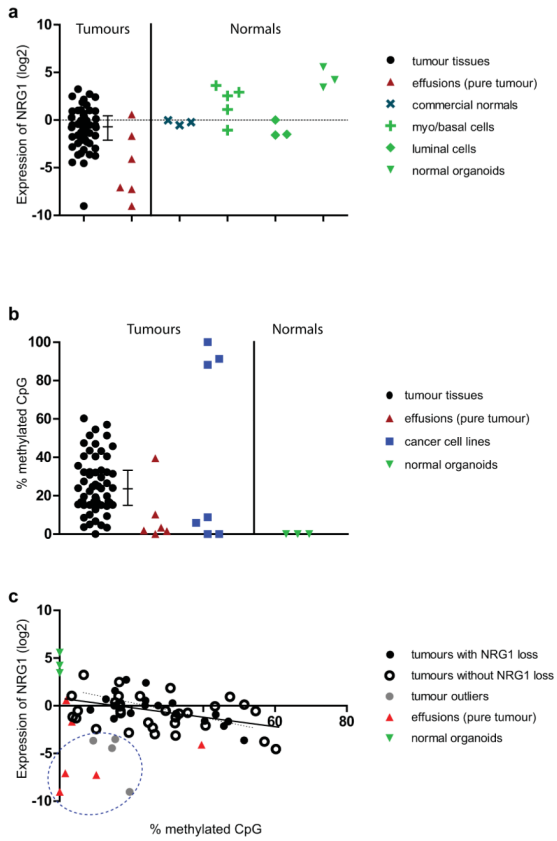

Figure 2.

NRG1 expression and DNA methylation in tumour and normal breast.

a NRG1 expression in tumours and various normal breast samples. NRG1 expression (as in Figure 1b) normalised to the average of all samples, log scale. Tumour tissues are the Nottingham series of breast tumour tissue samples, with median and inter-quartile values shown as lines adjacent to data points; 'effusions (pure tumour)' are pure breast tumour cells from five pleural and one ascitic effusions. Normals are RNA from normal breast tissue; myo/basal and luminal cells, respectively purified basal/myoepithelial and luminal cells from normal breast; and organoids, uncultured epithelial fragments from normal breast. **b** DNA methylation in tumours (with median and interquartile lines), purified tumour cells from effusions, and normal breast organoids, i.e. epithelial fragments prepared from normal breast. Representative cell lines are also shown, for comparison, reanalysed by pyrosequencing. Methylation, detected by pyrosequencing of uncloned bisulphite-treated DNA, is expressed as percent methylation averaged over the six CpGs pyrosequenced. **c** *NRG1* expression as a function of DNA methylation. The tumour tissue samples are divided according to whether they show genomic loss of 8p, including *NRG1*, by array-CGH. Dotted circle, 'outliers', i.e. samples with low expression in the absence of methylation (all outlier tumour tissue samples had 8p loss). The solid line is the regression line for all the tumour tissue samples, excluding the outliers (50 tumours, $y = -0.049 \times + 0.75$, $r \leq 0.19$, slope significantly less than zero, $p = 0.0017$); dotted line is for the samples with 8p loss excluding outliers (18 tumours, $y = -0.078 \times + 1.9$, $r \leq 0.42$, slope significantly less than zero, $p = 0.0036$).

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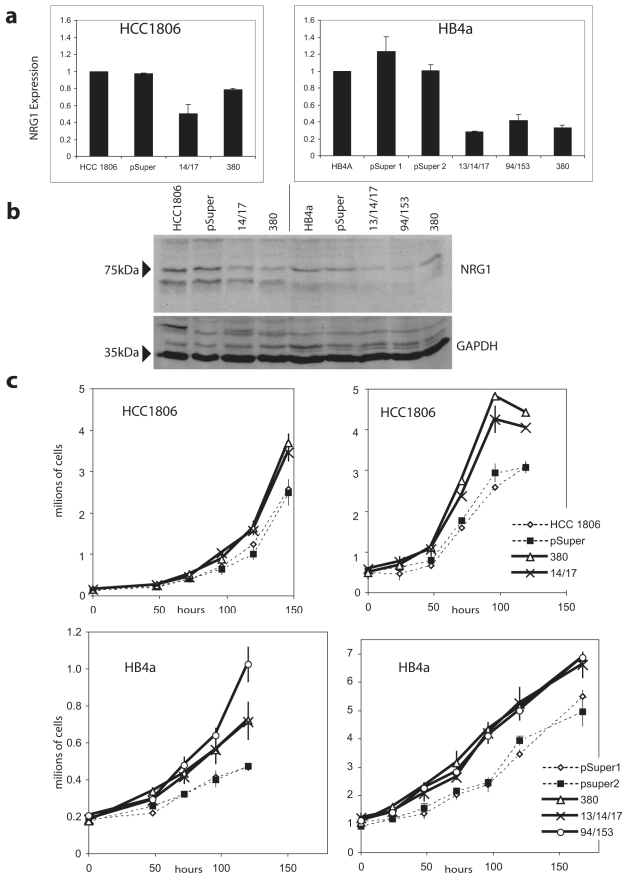

Figure 3.

Net cell proliferation is enhanced by knockdown of *NRG1*. Two cell lines that express *NRG1* were stably transfected with various siRNA constructs, resulting in a modest reduction in mRNA, typically around 50%. **a** downregulation of *NRG1* expression monitored by quantitative realtime RT-PCR (as in Figure 1). Samples are parental cells, pSuper vector-transfected controls, and different siRNA treatments identified by construct number, e.g. 13/14/17 indicates cells transfected with a mixture of constructs 13, 14 and 17. Error bars s.d. of triplicates. **b** Western blots of NRG1 protein and GAPDH control. **c** Growth curves. Cultures were seeded at 1.25×10^5 and 5×10^5 cells per 10 cm^2 well for HCC1806 derivatives and 2×10^5 and 1×10^6 for HB4a.

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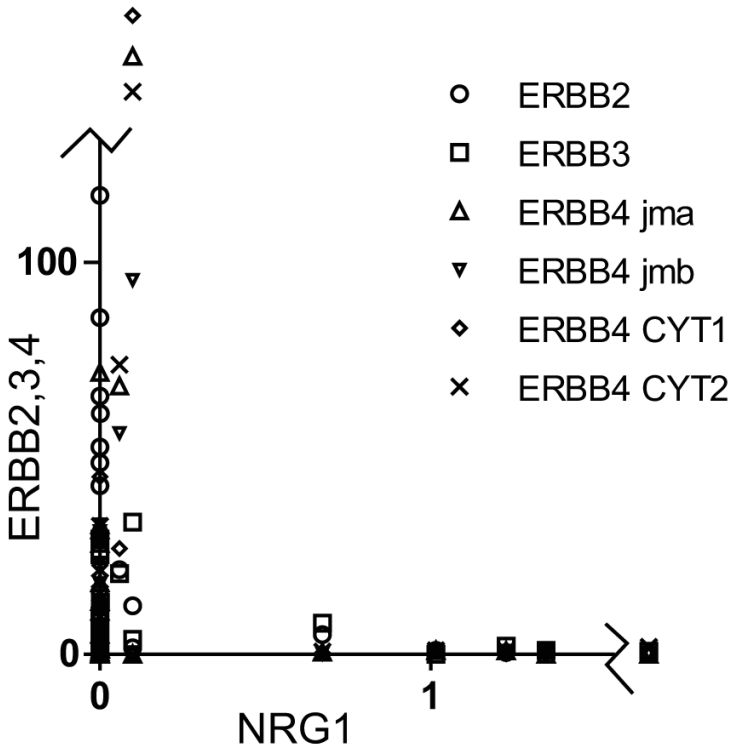

Figure 4.

Expression of *ERBB2*, *ERBB3* and *ERBB4* compared to expression of *NRG1*. As in Figure 1, expression levels were measured by real-time RT-PCR and are shown on a linear scale relative to expression in normal breast line HB4a, except for *ERBB3*, where expression is relative to non-cancer breast line HMT3522, since expression in HB4a is exceptionally low (8% of HMT3522). *ERBB4* expression was measured separately for each splice variant using isoform-specific primers: ERBB4 transcripts can include either the jma or jmb exon, though, in breast and breast cancer, the jma form predominates; and either include the CYT1 exon or skip it (giving CYT2 forms) (Muraoka-Cook et al., 2008; Sundvall et al., 2008). For complete data see Supplementary Figure 3. The data points off-scale for ERBB4 are for Cama-1, with values about 100 to 400 times HB4a levels; and the points off-scale for *NRG1* are for MDA-MB-415, with values four times HB4a.

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Table 1

Summary of *NRG1* status in breast cell lines

Cell line[a]	Origin	Classification[b]	Translocation break[c]		NRG1 expression[d]	NRG1 promoter methylation[e]
			present ?	position (Mb)		
HB4a	normal luminal		No		++	−
HMT3522	non-cancer		No		++	(−)
HCC1806	cancer		Yes	31.5	++	−
MDA-MB-175[a]	cancer	Lu	Yes	32.2	++	(−)
HCC1937	cancer	BaA	Yes	31.8	(+)	−
ZR-75-1	cancer	Lu	Yes	32.2	(+)	−
UACC812	cancer	Lu	Yes	31.8[f]	−	−
SUM52	cancer	Lu	Yes	31.4	−	−
HCC1569	cancer	BaA	Yes	32.1	−	+
MDA-MB-415	cancer	Lu	No		++	−
MDA-MB-157	cancer	BaB/M	No		−	−
HCC1187	cancer	BaA	No		−	(−)
HCC 1954	cancer	BaA/In	No		−	(+)
MDA-MB-134	cancer	Lu	No		−	+
BT474	cancer	Lu	No		−	+
HCC 1419	cancer		No		−	+
HCC 1500	cancer	Lu	No		−	+
MDA-MB-361	cancer	Lu	No		−	+
MDA-MB-453	cancer	Lu	No		−	+
T-47D	cancer	Lu	No		−	+
ZR-75-30	cancer	Lu	No		−	+

[a]MDA-MB-175 is the one line known to express a fused *NRG1* gene as a result of translocation. CAMA-1 is not shown as only expression was investigated for this line.

[b]Classification of cell lines into the subtypes of breast cancer defined by gene expression analysis, according to Charafe-Jauffret et al. (2006) and Neve et al. (2006): BaA, basal-like A; BaA/In, basal-like A or indeterminate; BaB/M, basal-like or mesenchymal-like; Lu, luminal.

[c] Translocation breakpoints in *NRG1* from Adelaide et al. (2003), Pole et al. (2006) and unpublished.

[d] Expression data summarised from Figure 1. ++, good expression, at least 50% of normal HB4a line; (+), detectable expression but much lower than normal.

[e] Methylation data summarised from Figure 1. +, many CpGs were over 50% methylated; (+), intermediate methylation; (−), some CpGs were 20 to 50% methylated.

[f] The breakpoint position for UACC812 is only approximate (Adelaide et al., 2003).