Membrane-Tethered Intracellular Domain of Amphiregulin Promotes Keratinocyte Proliferation

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The epidermal growth factor receptor (EGFR) and its ligands are essential regulators of epithelial biology, which are often amplified in cancer cells. We have previously shown that shRNA-mediated silencing of one of these ligands, amphiregulin (AREG), results in keratinocyte growth arrest that cannot be rescued by soluble extracellular EGFR ligands. To further explore the functional importance of specific AREG domains, we stably transduced keratinocytes expressing tetracycline-inducible AREG-targeted shRNA with lentiviruses expressing silencing-proof, membrane-tethered AREG cytoplasmic and extracellular domains (AREG-CTD and AREG-ECD), as well as full-length AREG precursor (proAREG). Here we show that growth arrest of *AREG*-silenced keratinocytes occurs in G2/M and is significantly restored by proAREG and AREG-CTD but not by AREG-ECD. Moreover, the AREG-CTD was sufficient to normalize cell cycle distribution profiles and expression of mitosis-related genes. Our findings uncover an important role of the AREG-CTD in regulating cell division, which may be relevant to tumor resistance to EGFR-directed therapies.

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INTRODUCTION

Amphiregulin (AREG) is one of seven members of a family of growth factors that bind to and activate the epidermal growth factor receptor (EGFR) (Sanderson et al., 2006). AREG was originally isolated as a secreted glycoprotein from the conditioned medium of phorbol-12-myristate-13-acetate-treated MCF7 human breast carcinoma cells (Shoyab et al., 1988). Independently, it was also purified from human keratinocyte-conditioned medium as a heparin-binding, autocrine factor (Cook et al., 1991) and as schwannoma-derived growth factor in mice (Kimura et al., 1990). AREG derives its name from the fact that it can either stimulate or inhibit growth of various normal and cancer cell lines cell lines (Johnson et al., 1991; Shoyab et al., 1988). Since its discovery, most publications about AREG have emphasized its growth-promoting and oncogenic activities (Busser et al., 2011). AREG is overexpressed in a wide spectrum of epithelial cancers, including breast (LeJeune et al., 1993; Qi et al., 1994), colon (Ciardiello et al., 1991), lung (Hurbin et al., 2002), ovary (Johnson et al., 1991), prostate (Bostwick et al., 2004), and squamous cell carcinomas of the head and neck (Dasgupta et al., 2006; Tinhofer et al., 2011) and skin (Rittie et al., 2007). Increased AREG expression levels correlate with poor response rates to the EGFR inhibitors gefitinib and cetuximab in non-small cell lung cancer (Busser et al., 2010a, 2010b; Ishikawa et al., 2005) and in head and neck squamous cell carcinomas (Tinhofer et al., 2011). Furthermore, AREG expression in T cells has been linked to $CD8^+$ T-cell-mediated tumor promotion (Kwong et al., 2010). Besides cutaneous squamous cell carcinoma, AREG has been implicated in the pathogenesis of psoriasis (Cook et al., 2004, 1997), keratoacanthoma (Billings et al., 2003), and retinoid irritation (Rittie et al., 2006). *AREG* mRNA is strongly induced in human skin organ culture (Stoll et al., 1997, 2010a) and plays an important role in the context of human keratinocyte proliferation and differentiation in vitro (Robertson et al., 2012; Stoll et al., 2010a, 2010b) and after xenotransplantation in vivo (Klingenberg et al., 2010).

The *AREG* gene in humans is located on chromosome 4q13.3 in a cluster with genes encoding three other EGFR ligands: epigen, epiregulin, and betacellulin. It encodes a 252 amino acid (aa) transmembrane precursor protein (proAREG) (Plowman et al., 1990). Metalloproteinase-mediated cleavage of proAREG near the N-terminus and near the outer leaflet of the cell membrane results in forms of soluble or shed AREG (sAREG) of 78 and 84 aa as well as a C-terminal domain (CTD) of 68–74 aa consisting of the juxtamembrane stalk (14–20 aa), transmembrane (23 aa), and cytoplasmic (31 aa) domains (Brown et al., 1998; Plowman et al., 1990; Sanderson et al., 2006) (see Supplementary Figure S1 online). Binding of proteolytically processed sAREG to EGFR has traditionally been thought to mediate its function by triggering the activation of key signaling pathways including ERK (Kansra et al., 2004; Stoll et al., 2010b)

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Abbreviations: aa, amino acid; Ab, antibody; AREG, amphiregulin; CTD, C-terminal domain; DAPI, 4′,6-diamidino-2-phenylindole; ECD, extracellular domain; EGFR, epidermal growth factor receptor; rh, recombinant human; sAREG, soluble/shed AREG; Tet, tetracycline

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and AKT (Gschwind et al., 2003). However, we found that growth arrest induced by *AREG* silencing could not be reversed by provision of recombinant soluble EGFR ligands or by expressing a form of the AREG extracellular domain (ECD) lacking a transmembrane domain (Stoll et al., 2010b). These surprising findings suggested the hypothesis that the AREG CTD might play a role in the regulation of keratinocyte proliferation. To explore this hypothesis in more detail, we stably transduced inducible *AREG* knockdown cells (Stoll et al., 2010b) with "silencing-proof" lentiviral expression constructs encoding transmembrane-tethered forms of the AREG-CTD and AREG-ECD and compared them with cells transduced with full-length proAREG. Because we have recently reported that keratinocyte growth inhibition after *AREG* silencing is mediated by mitotic arrest (Stoll et al., 2015), we have also explored the effects of the different AREG domains on cell cycle parameters and the expression of mitosis-related genes in this system. The results suggest an important signaling role of the AREG-CTD in driving keratinocyte mitosis.

RESULTS

We transduced previously established conditional *AREG* knockdown keratinocytes (N/TERT-TR-shAREG [Stoll et al., 2010b]; "parental cells") with lentiviral expression constructs encoding either full-length proAREG or membrane-tethered forms of AREG-ECD or AREG-CTD (Supplementary Figure S1). None of these three constructs is susceptible to shRNA-mediated silencing because the tetracycline (Tet)-inducible shRNA targets a sequence in the *AREG* 3′ untranslated region that is not present in these constructs.

After selection with puromycin, we assessed the expression of these constructs in the newly established cell lines by quantitative real-time PCR, using TaqMan gene expression assays that target nucleotide sequences in the AREG-ECD ("ECD assay") or the AREG-CTD ("CTD assay"). As shown in Figure 1, Tet treatment of parental cells reduced endogenous *AREG* transcripts by more than 95% (from 27% to 0.9% of the housekeeping gene *RPLP0* by the CTD assay and from 35% to 1.4% of *RPLP0* by the ECD assay). Tet treatment also decreased *AREG* mRNA levels in all three of these "AREG rescue" cell lines (AREG-CTD, AREG-ECD, and proAREG) relative to their corresponding no-Tet controls. Importantly, expression levels of all constructs in the Tet-treated AREG rescue cell lines were comparable with endogenous AREG levels in untreated control cells, demonstrating they produced physiologic levels of the various AREG constructs in the presence of Tet (20−38% versus 27−35% of *RPLP0*). Figures 1a and b also demonstrate the selective detection of the AREG-CTD and AREG-ECD by the corresponding TaqMan assays, whereas the proAREG construct is detected by both assays.

Using an ELISA in which the capture antibody (Ab) is directed against a recombinant form of sAREG, we tested the expression of cell-associated AREG protein in the various AREG rescue cell lines. As described previously (Stoll et al., 2010b), we found that cell-associated AREG protein expression in the parental cell line was reduced by more than 97% after 48 hours of Tet treatment (Figure 1c). AREG protein levels were also significantly reduced in the AREG-CTD rescue line, which does not express the AREG-ECD detected by the ELISA. In contrast, cell-associated AREG protein levels in the other two Tet-treated AREG rescue lines were similar to parental cells not treated with Tet (Figure 1c). Tet treatment also reduced sAREG levels in keratinocyte culture supernatants by 84.8% and 77% in the parental and AREG-CTD cell lines, respectively, and by 43% in the AREG-ECD cell line (Figure 1d). Next, we also examined AREG protein levels by immunostaining using an immunoaffinity-purified rabbit polyclonal Ab from Proteintech Group (PTG, Chicago, IL) raised against proAREG (the "PTG Ab"). Although this polyclonal Ab efficiently detects rhAREG (ECD from Ser101-Lys198; R&D Systems, Minneapolis, MN), it is unable to detect the 29-kDa GST fusion protein containing AREG aa Gln222-Ala252, indicating that the PTG Ab only recognizes ECD epitopes (see Supplementary Figure S2 online). As can be seen in Figure 1e, PTG Ab staining was predominantly localized in the perinuclear area and on the cell membrane as described (Higashiyama et al., 2008; Robertson et al., 2012; Stoll et al., 2015) and was markedly reduced in response to Tet in parental cells. In contrast, in the proAREG rescue cells treated with Tet, PTG Ab immunoreactivity was preserved at levels similar to parental cells without Tet. We also decorated these lines with an affinity-purified polyclonal Ab from Aviva (San Diego, CA) raised against a GST fusion protein containing the AREG-CTD (aa 222−252, the "Aviva Ab"). The Aviva Ab yielded relatively faint staining in parental cells, possibly due to the relatively low expression levels of AREG mRNA and protein in parental cells compared with the proAREG and AREG-CTD constructs (Figures 1a−d). However, and in contrast to the lack of nuclear staining detected by the PTG Ab, the Aviva Ab clearly yielded nuclear staining in the proAREG and AREG-CTD rescue cell lines (Figure 1e). Punctate cytoplasmic staining was also detected by the Aviva Ab in these two lines, albeit in a different pattern than the perinuclear staining detected by the PTG Ab. Also, in contrast to the near-total loss of staining by the PTG Ab after Tet treatment of parental and AREG-CTD cells, the nuclear and punctate cytoplasmic pattern of immunoreactivity detected by the Aviva Ab persisted in the presence of Tet in the proAREG and AREG-CTD rescue lines (Figure 1e).

Next, we tested these cell lines in autocrine growth assays (Stoll et al., 2015) in the presence or absence of Tet, with and without recombinant human (rh) AREG (100 ng/ml). A typical result is depicted in Figure 2a, and three to four independent experiments are quantified in Figure 2b. As expected, the Tet-inducible EGFP shRNA construct had no effect on cell counts, thereby ruling out a direct effect of Tet treatment on the outcome (Figure 2b). In agreement with our previous report (Stoll et al., 2010b), Tet-induced *AREG* knockdown in the parental cell line markedly reduced keratinocyte cell counts, to 8% of no-Tet controls. In contrast, the proAREG rescue construct substantially restored cell counts in the presence of Tet, to 55.8% of no-Tet controls. Notably, the AREG-CTD was nearly as effective as proAREG in restoring keratinocyte cell counts, to 47.7% of no-Tet controls, whereas AREG-ECD was much less effective, restoring growth to only 17.7% of no-Tet controls. Further analysis of cell counts (see Supplementary Table S1 online) showed a highly significant interaction of the effects of the various