Embryonic lethality in \textit{Dear} gene-deficient mice: new player in angiogenesis


Embryonic lethality in \textit{Dear} gene-deficient mice: new player in angiogenesis. \textit{Physiol Genomics} 23: 257–268, 2005; doi:10.1152/physiolgenomics.00144.2005.—The dual endothelin-1/angiotensin II receptor (\textit{Dear}) binds endothelin-1 (ET-1) and angiotensin II (ANG II) with equal affinities in the Dahl S/JR\textsuperscript{HS} rat strain. To elucidate its physiological significance within the context of multiple receptor isoforms and diverse ET-1 and ANG II functions spanning blood pressure regulation, tumor proliferation, and angiogenesis, we characterized mouse \textit{Dear} and \textit{Dear}-deficient mice. Unlike null mutant models of ET-1, ANG II, and all other ET-1 and ANG II receptors, \textit{Dear}\textsuperscript{−/−} deficiency results in impaired angiogenesis, dysregulated neuroepithelial development, and embryonic lethality by embryonic \textit{day} 12.5. Interestingly, mouse \textit{Dear} does not bind ANG II, similar to Dahl R/JR\textsuperscript{HS} rat \textit{Dear}, but binds ET-1 and vascular endothelial growth factor (VEGF) signal peptide (VEGFsp) with equal affinities, suggesting a putative novel multifunction for VEGFsp and a parsimonious mechanism for coordination of VEGF-induced and \textit{Dear}-mediated pathways. Consistent with its developmental angiogenic role, \textit{Dear} inactivation results in decreased tumor growth in \textit{B16-F10} melanoma cell-induced subcutaneous tumor in female \textit{Dear}\textsuperscript{−/−}/\textit{C57BL6}\textsuperscript{R10} mice, but not in males (age 3.5 mo), and in \textit{127Cs} radiation-induced orthotopic mammary tumors in Sprague-Dawley female rats (age range 3–6.5 mo). Altogether, the data identify \textit{Dear} as a new player in angiogenesis during development downstream to, and nonredundant with, VEGF-mediated pathways, as well as a putative modulator of tumor angiogenesis acting within a gender-specific paradigm.

Vascular Network Development, or vascularization, is a complex process whose key component paradigms, vasculogenesis, angiogenesis, and vascular remodeling, comprise interacting pathways involving or modulating vascular endothelial growth factor (VEGF)-A and its isoforms VEGF\textsubscript{121}, VEGF\textsubscript{165}, and VEGF\textsubscript{189}; angiopoietins 1 and 2 and their respective receptor tyrosine kinases; flk-1 or VEGFR2 receptor; and Tie2 or angiopoietin receptor (40). As with other processes, normal vascular development pathways are recruited into pathological pathways, producing a spectrum of pathological angiogenesis as seen in solid tumors, arthritis, and diabetes. While VEGF is a key regulator of vascularization in health and disease (7), the complexity of vascularization is nevertheless evident, as other key modulators of angiogenesis, defined by embryonic lethal phenotypes associated with abnormal embryonic and/or extraembryonic vascularization phenotypes, exist. These modulators represent diverse functional groups, such as transcription factors like hypoxia-inducible transcription factor (45) and HAND1 (34), energy metabolism regulators like Foxo1 (19), ion pumps like Na/Ca exchanger (9), integrins like β8 (58) and α7/β1 integrin (17) and regulators of integrins like focal adhesion kinase (49), growth factors like transforming growth factor (TGF)-B1 (31), signal transduction kinases like p38\textalpha mitogen-activated protein kinase (35) and G proteins like Go13 (44), and signal transduction modulators like Edd, a hyperplastic disc gene (48).

Additionally, cumulative observations add two vasoactive peptides, endothelin-1 (ET-1) and angiotensin II (ANG II), better known for blood pressure regulation, to the list of angiogenesis modifiers. Briefly, ET-1 is a potent vasoconstrictor peptide involved in diverse physiological functions such as blood pressure regulation, mitogenesis, apoptosis (30), and angiogenesis (46, 51) and has been implicated in several pathophysiological conditions such as hypertension, cardiac failure (25, 30, 53), and more recently tumor angiogenesis, invasion, and metastases (1, 20). Likewise, ANG II exhibits similar physiological responses to ET-1, such as blood pressure regulation, proliferation, apoptosis, and angiogenesis (54) and has also been implicated in hypertension (54), cardiac hypertrophy and failure (54), and tumor angiogenesis (15).

Concordant with these various functions, multiple single-ligand receptors have been characterized for both ET-1 and ANG II. For ET-1 receptor isoforms, ET\textsubscript{A} receptor detected in vascular smooth muscle cells binds ET-1 and -2 preferentially over ET-3, whereas ET\textsubscript{B} receptor detected in vascular endothelial cells binds ET-1, -2, and -3 equivalently (1). Relevant to angiogenesis, ET-1 and both ET\textsubscript{A} and ET\textsubscript{B} receptors have been associated with tumor angiogenesis (1). Likewise, of the three single-ligand ANG II receptors that have been characterized, AT\textsubscript{1A}, AT\textsubscript{1B}, and AT-2 receptors, AT\textsubscript{1A} underlies ANG II-stimulated angiogenesis in tumors (18), whereas AT\textsubscript{2} receptor negatively modulates ischemia-induced angiogenesis (50). Standing apart, however, is the dual ET-1/ANG II receptor (\textit{Dear}), which binds ET-1 and ANG II with equal affinities in the rat (43). \textit{Dear} responds to ET-1 and ET-2 but minimally to ET-3 (43), just like the ET\textsubscript{A} receptor. Recent studies further elucidate that the dual-ligand status is specific for S44/M74 Dear detected in Dahl salt-sensitive, hypertensive (S)/JR\textsuperscript{HS} rat strains, spontaneously hypertensive, Wistar Kyoto and Brown Norway rat strains (28). A \textit{Dear} variant detected in Dahl R and Lewis rat strains, S44/P/M74 Dear, binds ET-1 but not ANG II (28).

When one reviews phenotypes of gene deficiency mouse models for ET\textsubscript{A}, ET\textsubscript{B}, AT\textsubscript{1A}, AT\textsubscript{1B}, and AT2 receptors, the lack of angiogenesis-associated phenotypes, despite multiple functional studies implicating ET-1 and ANG II in pathological angiogenesis (1, 18), indicates complexities in angiogenic

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paradigms. More specifically, gene-targeted deficiencies of ET-1 and its receptors, ET\textsubscript{A} and ET\textsubscript{B}, as well as angiotensinogen (Agt) and all ANG II receptors, AT\textsubscript{1a}, AT\textsubscript{1b}, and AT\textsubscript{2}, do not result in embryonic lethal angiogenesis deficits (8, 10, 22, 24, 26, 29, 36, 52). Moreover, reported null embryo phenotypes do not reflect reported angiogenesis-associated functions: severe craniofacial abnormality and postnatal respiratory failure in ET\textsubscript{A}−/− (29) and in ET\textsubscript{A}−/− deficiency (10), angionic megacolon in ET\textsubscript{B}−/− deficiency (24), hypotension and renal abnormalities in Agt−/− deficiency (36, 52), absent ANG II pressor response in AT\textsubscript{1a}−/− deficiency (26), increased ANG II pressor response in AT\textsubscript{2}−/− deficiency (22), and no reported abnormality in AT\textsubscript{1b}−/− deficiency (8). Even double AT\textsubscript{1a}−/−/AT\textsubscript{1b}−/− receptor deficiency does not elicit an embryonic lethal angiogenesis phenotype (37). An indirect association with tumor-associated, pathology-induced angiogenesis through interactions with the VEGF axis is apparent, however, identifying the AT\textsubscript{1a} receptor as proangiogenic (14, 47). Although one could deduce that angiogenic properties of ET-1 and ANG II are mediated through induction or modulation of VEGF (18, 38), an alternative hypothesis is that Dear may mediate observed angiogenic properties of ET-1 and/or ANG II.

On the basis of these observations, we tested the hypothesis that, as a dual receptor for ET-1 and ANG II, Dear (43) may directly mediate the angiogenic role of ET-1 and/or ANG II. Here we show that Dear−/− deficiency results in impaired vasculogenesis, aborted angiogenesis, and dysregulated neuro-epithelial development. We also show that mouse Dear binds ET-1 and VEGF signal peptide (VEGFsp) with equal affinities, but not ANG II, suggesting a novel function for VEGFsp concordant with the emerging concept of multifunctional signal peptides. More importantly, Dear-mediated effects on vascular network formation translate to reduced tumor growth in melanoma and mammary tumor rodent models upon inhibition of Dear-mediated pathways.

**MATERIALS AND METHODS**

**Characterization of 129SVJ mouse Dear gene.** We isolated a genomic clone for mouse Dear from a XFIXII 129SVJ mouse genomic library screened with the full-length 3,274-bp rat Dear cDNA (43) as probe. Six independent genomic clones were identified and plaque purified; one clone, A191, was characterized further by restriction digestion and subsequent Southern blot analysis. A single 8-kb BamHI/BamHI restriction fragment (that hybridized to the 3,274-bp rat Dear CDNA probe) was subcloned into psp73 plasmid vector and sequenced.

**Characterization of mouse Dear cDNA and expression studies.** Mouse Dear cDNA was obtained by RT-PCR from C57BL/6 mouse kidney PolyA+ RNA (forward primer: 5′-CACCACAAGGCTTTAATTCTTATCC-3′; reverse primer: 5′-AAAGCCAGCCCTTGATGATAACC-3′), subcloned into the PT-vector system (Clontech, Palo Alto, CA), and then sequenced (GenBank accession no. DQ009865). The DNA blot analysis was done as described (43), using PolyA+ RNA (3 μg) and 32P end-labeled anti-sense mouse Dear oligonucleotide (5′-AGTGATAGAGCCCCAGTCTTCAGACTATCTCTCTTG-3′) as probe. Receptor expression studies with 125I-ET-1 and 125I-ANG II binding to membranes were done as described (28). Displacement of 125I-ET-1 binding was used to determine relative binding affinities for VEGF and VEGFsp to mouse Dear expressed in transfected Cos-1 cells. VEGFsp was synthesized from the following peptide sequence: NH\textsubscript{2}-MNFLLSVWHTLALLYLHHA-KWSQA-COOH.

**Targeted disruption of Dear in mice.** Animal protocols were approved by our Institutional Animal Care and Use Committee. We performed all animal procedures in accordance with institutional guidelines. We constructed the targeting vector by replacing a 300-bp piece containing the 3′-end of Dear with the PGKneo cassette (see Fig. 1D), thus deleting amino acids 81–127 of Dear. We detected targeted Dear disruption by Southern blot analysis of Sp\textsubscript{II} digest genomic DNA using a 1.5-kb Dear fragment as probe (see Fig. 1D). Detection of a 5.2-kb fragment indicates homologous recombination in contrast to an 8-kb endogenous fragment (Fig. 1). Homologous recombination was further verified by PCR analysis using an upstream primer (P1: 5′-TGTGAGGCTAGAAGGCTGC-3′) located 171 bp upstream from the 5′-end of the targeting vector and a reverse primer (P2: 5′-GAGCAAGGTTGAGATGACCGG-3′) located in the PGK-neo cassette (Fig. 1). Amplification of a 5.5-kb fragment that hybridized to the same probe used in the Southern blot analysis (Fig. 1) was indicative of homologous recombination. Of five positive embryonic stem (ES) cell clones, we microinjected two clones into 129SVJ blastocysts, generating 14 chimeric mice; subsequent germ-line transmission of targeted allele established the Dear knockout line. Speed congenic backcross breeding to inbred onto C57BL/6 genetic background was done for >10 generations, ΔDear/C57BL6, providing all Dear−/− and Dear+/− mice for analyses (>99.9% congenic line in C57BL/6 background).

**Genotyping of mouse embryos.** We genotyped embryonic yolk sacs by PCR analysis of genomic DNA using primers flanking the SacI site.
localized within the amino acid coding region of Dear (upstream primer: 5′-AACTTTCTCCTGTCGGCCCTC-3′; downstream primer: 5′-ACTTGCTGAAACTAAACCTGC-3′); wild-type allele results in 153-bp PCR product, and disrupted allele results in 5.5-kb PCR product (Fig. 1F).

Embryo analysis, histology, and immunohistochemistry. We analyzed embryos collected at embryonic day 9.5 (E9.5) through E12.5 from timed-pregnant mice and determined genotypes by PCR analysis of yolk sac-derived DNA. Anatomic analysis of embryos comprised stereomicroscopic analysis and digital photography with and without yolk sacs, followed by immersion fixation in PBS-buffered 4% paraformaldehyde, pH 7.8. Histology processing and staining were done following established procedures for paraffin-embedded sections; immunohistochemistry was done as described (23).

Analysis of heterozygous Dear+/– phenotype. We analyzed backcross BC10(C57BL/6) Dear+/– mice for Dear protein levels by Western blot analysis, using equal amounts of protein (40 μg) from mouse kidney membranes isolated as described (27) and rabbit IgG anti-mouse Dear anti-peptide-specific antibody (1:500 dilution, 16 h at 4°C) developed against mouse Dear specific synthetic peptide: L155SKCNHNEQDTAT27, to detect Dear-specific polypeptide. Densitometry analysis was done for quantification; readings were normalized to common nonspecific peptide in each lane and background.

We measured blood pressure (BP) in 6-mo-old mice by tail-cuff sphygmomanometer (Vitaltech BP 2000) under light anesthetic (for males, 50% of anesthetic dose equal to 0.018 mg/g body wt xylazine, 0.105 mg/g body wt ketamine; for females, 37.5% of full anesthetic dose) while ascertaining equivalent physiological state by anesthetic dose (55) in 12-wk-old range.

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Tumor studies and Dear-specific inhibition. We developed B16-F10 (ATCC) melanoma cell-induced subcutaneous tumor model as described (55) in 12-wk-old Dear+/– and littermate Dear+/+ male and female mice (n = 5/group) maintained on regular mouse chow. Thirteen days after tumor induction (3.5 mo of age), we excised tumors and measured tumor weight and volume. We induced rat mammary gland tumors in 48 Sprague-Dawley rats on regular rat chow (n = 12/group) as described (11) at 40 days of age via 127Cs irradiation. All rats were maintained on regular rat chow. Only rats with heart rate ranging from 300 to 500 beats/min (bpm). We obtained 3 sets of 10 consecutive readings per mouse and took the average of at least 20 readings within the prescribed normal heart rate range.

RESULTS

Characterization of mouse Dear and development of Dear-deficient mice. To investigate Dear function, we characterized and inactivated mouse Dear gene by gene targeting experiments in mice (Fig. 1), thus allowing comparative analysis to published knockout mouse models of ET-1, ANG II, and their respective multiple receptors. Molecular characterization of mouse Dear genomic clone detects a one-exon transcription unit showing 78% amino acid sequence homology with the rat receptor (43) (Fig. 1A). Sequence differences in the predicted ANG II binding site correlate with mouse Dear binding studies showing nondual activation: mouse Dear binds solely to ET-1 [dissociation constant (Kd) ET-1 = 0.776 ± 0.064 nM, maximum binding capacity (Bmax) = 13.02 ± 1.27 fmol/mg; Fig. 1B]. This molecular phenotype resembles the recently characterized Dahl R Dear S44P/M74T rat variant, which responds solely to ET-1 as well (28). These data suggest that observations in Dear-deficient mice are most likely not ANG II mediated. RNA analysis detects mouse Dear mRNA in all tissues tested, with the highest level of expression in kidney and aorta (Fig. 1C). Targeted inactivation of Dear–/– in ES cells results in the deletion of the last 47 amino acids of the Dear polypeptide, including the putative G protein-interacting domain (43) (Fig. 1D). Southern blot analysis (Fig. 1E) and PCR amplification of homologous recombination-specific event (Fig. 1F) confirmed gene-targeting events in ES cell clones and subsequent Dear-deficient progeny. Heterozygous Dear-deficient (Dear+/–) male and female mice (backcross-10 inbred C57BL/6 mouse strain) exhibit significantly less Dear protein in mouse kidney protein blot analysis, as detected using an anti-mouse Dear anti-peptide-specific antibody (Fig. 1G; P < 0.05, t-test), less body weight at 5 and 6 mo of age (males: P = 0.007; females: P = 0.0006; Fig. 1H), and decreased BP in female (BP mean ± SD; Dear+/– at 134 ± 40 mmHg vs. Dear–/– at 112.3 ± 6.1; P < 0.01) but not male mice while heart rate remains equivalent (Fig. 1H). Gender-specific BP effects are concordant with observations in a recent study of rat genetic hypertension wherein Dear variants cosegregated with hypertension in female but not in male F2-intercross rats (28).

Dear–/– deficiency results in embryonic lethality by E12.5. PCR-based analysis of progeny from heterozygous Dear+/– male and female crosses detected 29 wild-type (Dear+/+), 39 heterozygous (Dear+/–), and 0 null (–/–) mice derived from 17 litters. The absence of null genotypes in all live births demonstrates that Dear null mutation is embryonic lethal. To investigate embryonic lethality in Dear–/– embryos, we analyzed embryos at different stages of development. From 129 E9.5–E12.5 embryos, we detected 33 (–/–), 67 (+/–), and 29 wild-type (+/+ ) genotypes (Fig. 1F) conforming to the expected 1:2:1 segregation ratio for a standard (+/–) × (+/+) intercross. We detected embryonic lethality around E12.5.

Abnormal vascular network formation and morphology in Dear–/– deficient embryos. Analysis of E12.5–E10.5 embryos revealed absent yolk sac collecting vessels associated with homozygous Dear–/– deficiency (Fig. 2, A–F); heterozygous Dear+/– deficient embryos exhibited normal yolk sac vascularization (data not shown). We detected hemorrhagic, resorbed embryos as early as E10.5 but mostly at E12.5 (Fig. 2D). Smaller and strikingly paler than Dear+/+ and Dear–/– embryos, Dear–/– embryos exhibit two size phenotypes: a
dysmorphic phenotype detected from E10.5 to E12.5 (Fig. 2, A–C) that is relatively larger than a second hypoplastic phenotype (Fig. 2, E–F). To determine whether genetic variation influences the null phenotype, speed congenics were done onto C57BL/6 genetic background, and backcross-10 null mice were generated and analyzed confirming embryonic range of lethality, absent yolk sac collecting vessels, and both dysmorphic and hypoplastic embryo phenotypes in Dear⁻/⁻ embryos (Fig. 2, A–F). Analysis of dysmorphic Dear⁻/⁻ embryos at E10.5 and E11.5 detected blood-filled hearts (Fig. 2, G–H) that contracted, despite impaired vascular formation typified by disorganized, blood-filled pools in the cranial region without apparent connection to a blood-filled heart (Fig. 2G) or minimal vascular networks in both cranial and caudal regions and a dilated blood-filled heart (Fig. 2H). This contrasts with the prominent vascular network marked by blood-filled dorsal aorta and blood vessels in the cranial region that are characteristic features of E9.5 Dear⁺/+ embryos (Fig. 2G). Furthermore, analysis of fixed E11.5 embryos revealed abnormal brain and cardiac morphology in Dear⁻/⁻ embryos (Fig. 2I).

Dear⁻/⁻ deficiency alters angiogenesis and cardiac and neuroepithelial development. Histological analysis of Masson-trichrome-stained E10.5–E11.5 embryo sections confirm minimal-to-absent collecting vessels in the yolk sac in Dear⁻/⁻ embryos compared with Dear⁺/+ embryos (Fig. 3A). We detected an attenuated yolk sac primary vascular plexus in Dear⁻/⁻ embryos, along with a markedly reduced number of blood islands in Dear⁻/⁻ embryos compared with Dear⁺/+ embryos (Fig. 3A). Despite absent yolk sac collecting blood vessels, E11.5 Dear⁻/⁻ embryos exhibit fetal-placental vascular connection, albeit markedly hypoplastic, in contrast to Dear⁺/+ littermate embryos (Fig. 3A). Additionally, we detected reduced placental fetal-maternal vascular plexus in Dear⁻/⁻ embryos in contrast to robust blood-filled fetal-maternal vascular plexus in Dear⁺/+ embryos (Fig. 3A). In contrast to minimal blood islands in the yolk sac, we detected

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scattered blood islands in Dear<sup>−/−</sup> embryos but markedly underdeveloped dorsal aorta and peripheral vasculature, suggesting deficiencies in primary vascularization despite the presence of blood islands (Fig. 3B). Analysis of cardiac development in adjacent littermates revealed that Dear<sup>−/−</sup> hearts have rudimentary cardiac chamber formation and absent endocardial cushion formation (Fig. 3B). Analysis of neural tube development revealed markedly convoluted neuroepithelium in the brain (Fig. 3B) and spinal cord (data not shown). In the fore- and midbrain regions, apparent left-right symmetry prevailed despite neuroepithelial convolutions (Fig. 3B), thus suggesting a developmental process gone awry rather than a degenerative process due to deficient vascular network development. Histological analysis at E12.5 revealed persistence of disordered neuroepithelial growth, resulting in convolutions throughout the brain and spinal cord, as well as a lack of endodermal-derived organogenesis in Dear<sup>−/−</sup> embryos in contrast to littermate Dear<sup>+/+</sup> controls (Fig. 3E). Closer analysis revealed large areas of embryonic blood cells in the ventral midportion that were not contained in blood vessels or in recognizable liver tissue (data not shown). Counterintuitive to a hypercellular, convoluted neuroepithelium, we detected rudimentary, thin-walled blood vessels in the perineural regions with sparse blood cells in Dear<sup>−/−</sup> embryos, in contrast to Dear<sup>+/+</sup> embryos, which exhibited perineural blood vessels filled with nucleated blood cells (Fig. 3C). Concurrent with sparse perineural vessels, only a few penetrating capillaries are evident in Dear<sup>−/−</sup> embryo neuroepithelium in contrast to Dear<sup>+/+</sup> embryo (Fig. 3C).

In further analysis of vascular deficits, immunohistochemical staining for smooth muscle cell α-actin revealed scattered expression in E12.5 Dear<sup>−/−</sup> embryos and intense staining in the embryo-placenta vascular connection (Fig. 3D). Perineural blood vessels exhibited α-actin immunostaining in Dear<sup>−/−</sup> embryos but had minimal angiogenic branching in contrast to Dear<sup>+/+</sup> embryos wherein angiogenic sprouting is evident (Fig. 3D). Closer histological analysis revealed sporadic blood islands incompletely circumscribed by α-actin-stained single cell vascular wall in Dear<sup>−/−</sup> embryos (Fig. 3D).

Concordance of Dear gene embryonic spatial expression pattern with null phenotype. To investigate mouse Dear temporal and spatial expression patterns, we analyzed Dear expression in E9.5–E12.5 wild-type embryos using an anti-mouse Dear peptide-specific antibody validated to detect Dear polypeptide (Fig. 1G). At E9.5, we detected Dear expression predominantly in the heart, yolk sac mesodermal layer and endothelium, fetal vascular endothelium in the placenta, dorsal aorta, and ependymal layer of the neural tube (Fig. 3E). These expression patterns persisted at E12.5, where we detected increased expression in some hemangioblasts in yolk sac blood islands (Fig. 3E), as well as more prominent expression in the ependymal layer of the neuroepithelium and in perineural blood vessel walls (Fig. 3E). Observed temporal and spatial expression patterns are concordant with vascular, cardiac, and neuroepithelial phenotypes observed in Dear<sup>−/−</sup> deficient mice.

VEGFSp binds Dear with high affinity and competes with ET-1 binding. Because of the striking differences in phenotypes of Dear<sup>−/−</sup> and ET-1<sup>−/−</sup> (29) deficient mice, we investigated putative novel Dear ligands among genes whose gene-targeted deficiencies result in embryonic lethality due to abnormal angiogenesis. We next tested which of these gene products are predicted to bind Dear as an ET-1 mimetic based on a modified molecular recognition theory that predicts that binding domains and cognate ligands evolve from complementary DNA strands, both of which can be encoded by any of the three possible translational frames of said DNA sequence (3, 42). Quite interestingly, structural comparison of the VEGFSp region and ET-1 reveals 66% nucleotide (nt) sequence identity in the ET-1 nt region no. 31–59, with striking similarity between the third reading frame of ET-1 with VEGFSp peptide (Fig. 4A). On the basis of the molecular recognition theory, these similarities would suggest that VEGFSp is a putative mimetic ligand of ET-1 and hence could be expected to bind to the predicted ET-1 binding site on Dear. Analysis of VEGFSp binding affinities of transfected Cos-1 cell membranes expressing functional mouse Dear (Fig. 1B) revealed high-affinity binding of VEGFSp (EC<sub>50</sub> = 4.56 ± 0.33 nM) to Dear equivalent to ET-1 (EC<sub>50</sub> = 4.82 ± 0.28 nM) in contrast to nonbinding of VEGF (Fig. 4B) and ANG II (Fig. 1B). These data suggest the hypothesis that VEGFSp could underlie the developmental angiogenesis phenotype observed in Dear<sup>−/−</sup> deficient mice, thus accounting for the nonangiogenic, nonembryonic lethal phenotype of ET-1<sup>−/−</sup> deficiency (29).

Dear inhibition reduces tumor growth and alters promalignant parameters. On the basis of these observations and the emerging role of VEGF and ET-1 in tumor angiogenesis, we investigated the role of Dear inhibition in two established rodent tumor models. First, comparing heterozygous Dear<sup>−/+</sup> deficient mice and wild-type Dear<sup>+/+</sup> littermates, we detected significant reduction in tumor mass (Fig. 5A) and tumor volume (Fig. 5B) in the B16-F10 melanoma cell-induced tumor model (55) in heterozygous Dear<sup>−/+</sup> deficient female mice (2-tailed t-test, P < 0.02) but not male mice. Second, because effects were seen only in female Dear<sup>−/+</sup> mice, we next tested whether Dear inhibition would reduce tumor growth in the 125I<sup>+</sup>Cs radiation-induced breast cancer model (11) in female rats with tumor latency less than 3 mo. Using two independent inhibition methods, anti-rat Dear peptide-specific antibody (ab-Rx) begun 4 wk after irradiation (Fig. 5C) and anti-rat Dear DNA vaccine (DNA-v) begun 2 wk after irradiation (Fig. 5D), we detected significant reductions in tumor growth during a 6-wk observation period. In contrast
to respective control groups, both anti-Dear treatments prevented tumor growth with significant reductions in percent change in tumor volume detected from 4 to 6 wk after tumor appearance (ab-Rx: ANOVA \(\times\) Tukey's test, \(P < 0.05 - 0.01\); DNA-v: ANOVA \(\times\) Tukey's test, \(P < 0.02 - 0.001\)). Furthermore, we detected significant tumor regression with 68% reduction in tumor volume in anti-Dear DNA-vaccinated rats (Fig. 5D; ANOVA \(\times\) Tukey's test, \(P < 0.01\)).

Histopathological analysis of Masson-trichrome-stained tumor sections revealed that decreased tumor size in both treatment groups was associated with large areas of fibrosis (data not shown). In regions with tumor cells, stromal invasion was observed in both treated and control groups, but tumors from both treatment groups exhibited lower promalignant potential (Fig. 5E) as follows. High-magnification analysis of tumor vasculature revealed vascular invasion by tumor cells in contrast to minimal, if any, vascular invasion or mosaic vessels in both anti-Dear treatment groups (Fig. 5E, middle). High-magnification analysis of tumor cell nuclei detected greater nuclear pleomorphism with greater variation in size, shape, and hyperchromasia in both control groups compared with both anti-Dear treatment groups (Fig. 5E, right). These data suggest that tumor size and vascular and nuclear promalignant phenotypes are attenuated by inhibition of Dear-mediated pathways.

**DISCUSSION**

Although “Dear” is a misnomer for mouse Dear, since it is a dual ET-1/VEGFsp/non-ANG II receptor, analysis of Dear\(^{-/-}\) deficient mice reveals that Dear-mediated signaling is necessary for angiogenesis and vascular network development.

Compared with VEGF\(^{-/-}\) and VEGF\(^{+/+}\) deficient embryos (6, 16), the later embryonic lethality and detection of primary vascularization but aborted vascular expansion and networking in Dear\(^{-/-}\) deficient embryos suggest that the angiogenic role of Dear-mediated pathways is downstream to VEGF-mediated pathways, albeit equally important to functional vascularization and embryo viability.

The finding that Dear\(^{-/-}\) deficiency results in embryonic lethal vascular network abnormalities, whereas ET-1\(^{-/-}\) inactivation does not interfere with vascular networking (29), is puzzling and suggests an alternative gene source(s) for ET-1 or the existence of ET-1 mimetic ligands. The latter possibility is concordant with our observations, suggesting the hypothesis that VEGFsp, which binds Dear with equal affinity compared with ET-1, could underlie Dear-mediated angiogenic roles in development. This putative new role of VEGFsp as the ET-1-like ligand for Dear predicted by the molecular recognition theory is concordant with emerging evidence demonstrating that signal peptides have multifunctional biological roles beyond the signal peptide-targeting function (21). Binding of VEGFsp to Dear would provide a putative parsimonious mechanism for direct coordination between the VEGF system and Dear: as proVEGF is clipped, its signal peptide, VEGFsp, binds Dear, subsequently prompting modulation and/or activation of Dear-mediated pathways. This notion parallels the emerging diverse roles of signal peptides such as 1\(^{st}\) immuno-recognition modeled by the binding of signal sequences of certain polymorphic major histocompatibility complex (MHC) class I molecules to human lymphocyte antigen (HLA)-E and presentation as antigen on the cell surface by HLA-E with the
peptide-HLA-E complex interacting with natural killer cells (4, 5); 2) potential modulation of signal transduction as modeled by NH2-terminal portions of signal peptides of preprolactin and human immunodeficiency virus-1 gp160, which are, respectively, released to the cytosol and bind to calmodulin in a Ca2+-dependent manner (33); and 3) a putative role in neuro-modulation of body weight by neuropeptide Y signal peptide through a gain-of-function polymorphism (12). Although more studies are necessary to dissect ligand-specific roles and multiligand hierarchical relationships for Dear activation, high-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

**Fig. 5. Analysis of Dear inhibition on tumor growth.** A and B: in Dear+/− mice (■), decreased tumor mass (A; in mg) and tumor volume (B; in mm3) of melanoma cell-induced subcutaneous tumors was observed in females but not males compared with age-matched Dear−/− control mice (□) (2-tailed t-test). C: anti-rat Dear anti-peptide-specific antibody treatment (○) results in decreased tumor volume in radiation-induced rat mammary tumors (ANOVA + Tukey’s test). D: anti-rat Dear DNA vaccine treatment (○) also results in decreased tumor volume in radiation-induced rat mammary tumors (ANOVA + Tukey’s test). E: representative histological analysis of Masson-trichrome-stained tumor sections comparing mock-treated (mock-Rx) vector controls, anti-Dear anti-peptide-specific antibody treatment (ab-Rx), and anti-rat Dear DNA-vaccine (DNA-vac) shows changes in tumor pattern, microvascular invasion, and nuclear grade in anti-Dear-treated tumors (bar = 20 μm). Values are means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001.
affinity binding of VEGFsp to Dear suggests the novel hypothesis of VEGFsp’s multifunctionality as a ligand for Dear-mediated function(s).

The convoluted hypercellular neural tube detected in Dear−/− deficient embryos at E11.5–E12.5 has not been described before, thus exposing a novel developmental paradigm for the brain and spinal cord. Moreover, given that both Dear−/− and VEGF−/− deficiencies result in sparse perineural vessels and absent penetrating capillaries in the neuroepithelium, the convoluted hypercellular neuroepithelium in Dear−/− mice contrasts the observed increase in neuroepithelial apoptosis and thinning seen in heterozygous VEGF+/− deficient mice (16) and in nestin-cre/VEGFlox−/− neuron-targeted VEGF-deficient mice (39). This contrast, coupled with the detection of increasing Dear expression in the ependymal layer of the neuroepithelium from E9.5 to E12.5 (Fig. 3E), suggests a Dear-specific role in neuroepithelial development, although currently the lack of vascular-neural interaction cannot be ruled out as causal, reminiscent of parallel vessel sprouting and axonal arborization in development (7).

The significant inhibition of tumor growth by anti-Dear treatment in radiation-induced orthotopic mammary tumors in female rats is concordant with the association of ET-1 with angiogenesis in breast cancer (56). Although ET-1 effects on tumor angiogenesis are attributed to modulation of VEGF (56), identification of Dear as a new player in angiogenesis provides a direct pathway for ET-1 effects in tumor angiogenesis. The attenuation of nuclear pleomorphism and polychromasia are also concordant with the association of ET-1 with promalignant potential in breast cancer (57). While tumorigenic ET-1 effects are well characterized (a logical deduction can be made that anti-Dear effects on tumor angiogenesis and malignant potential are ET-1 mediated), the tumorigenic properties of VEGFsp have not been described to date, and hence VEGFsp cannot be ruled out as a possible ligand for this Dear-mediated function. Furthermore, the hierarchical relationship of ET-1 and VEGFsp in tumor angiogenesis and malignant potential remains to be elucidated. Alternatively, because both bind Dear with equivalent affinities and compete for the same binding site (Fig. 4), VEGFsp and Dear and ET-1 Dear activation may form redundant/versatile pathways for tumor angiogenesis. Nevertheless, confidence in the anti-tumorigenic effects of anti-Dear treatment is supported by the observed combinatorial effects: decrease in tumor size and attenuation of promalignant potential measured as attenuation of nuclear pleomorphism, polychromasia, and vascular invasion or mosaic tumor microvasculature.

The gender-specific effects of Dear inhibition in induced subcutaneous melanoma in Dear−/− female mice, corroborated in orthotopic rat mammary tumors, recapitulate the gender-specific effects of Dear variants in salt-sensitive hypertension (28). While elucidation of mechanisms requires further study, current information suggests the following plausible mechanisms: estrogen-specific transcriptional regulation through estrogen response elements in the 5′-flanking regulatory region and/or in the transcriptional unit per se (potential estrogen response element is detected within the rat and mouse Dear transcription units; unpublished observations), estrogen-specific modulation of signal transduction mechanisms (13), or a priori gender-specific hardwiring (cellular and molecular environments), which differentially affects mechanisms involved in tumor pathogenesis. We note, however, that while gender-specific effects are clear in hypertension (28) and in tumorigenesis, a gender-specific effect is not evident in embryonic lethality, since there are no live Dear−/− deficient pups born. This may be attributed to the fact that, before the formation of the genital ridge at E12 with detectable gender-specific features, gonad primordia are indistinguishable as to sex up to E11.5 (41), at which time processes leading to embryonic lethality of Dear−/− pups are already underway, since embryonic lethality from Dear−/− deficiency is observed from E10.5 to E12.5.

Altogether, while ligand-specific functions and hierarchical relationships remain to be elucidated for this multigand receptor, making its name a misnomer, the data identify Dear as a new player in angiogenesis with a significant role in cardiovascular and neural development as well as in tumorigenesis. Parallel to observations in salt-sensitive hypertension (28), the data also identify Dear as an effector of gender-specific modulation of tumor disease course, although specific mechanisms remain to be elucidated. More importantly, Dear as a new player in angiogenesis with an embryonic lethal phenotype demonstrates that our current understanding of angiogenesis is incomplete and reiterates the complexity of vascular network formation in development and disease.

DISCLOSURES

We declare that we have no competing financial interests.

REFERENCES


