H-, N- and Kras cooperatively regulate lymphatic vessel growth by modulating VEGFR3 expression in lymphatic endothelial cells in mice
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SUMMARY
Mammalian Ras, which is encoded by three independent genes, has been thought to be a versatile component of intracellular signalling. However, when, where and how Ras signalling plays essential roles in development and whether the three Ras genes have overlapping functions in particular cells remain unclear. Here, we show that the three Ras proteins dose-dependently regulate lymphatic vessel growth in mice. We find that lymphatic vessel hypoplasia is a common phenotype in Ras compound knockout mice and that overexpressed normal Ras in an endothelial cell lineage selectively causes lymphatic vessel hyperplasia in vivo.

INTRODUCTION
Three highly related GTPases, H-, N- and Kras, encoded by the three mammalian Ras genes, have been thought to play pivotal roles in the intracellular signalling pathways that regulate cell proliferation, survival and differentiation (Karnoub and Weinberg, 2008). Their intrinsic GTPase activity is regulated or modulated by a balance between Ras guanine nucleotide exchange factors and Ras GTPase activating proteins in response to ligand/receptor signals. GTP-bound Ras acts as an ‘ON’ switch by binding its effector proteins and activating downstream signalling pathways such as Raf/MAPK and phosphoinositide 3 (PI3) kinase/Akt (Karnoub and Weinberg, 2008).

It has been well documented that hyperactivation of mutated Ras proteins is responsible for human diseases. In addition to findings of gain-of-function characteristics of mutated Ras proteins deeply involved in tumorigenesis (Karnoub and Weinberg, 2008), germline gain-of-function mutations for the HRAS, NRAS and KRAS genes have recently been found in individuals with Costello, cardio-facio-cutaneous and Noonan syndromes, which exhibit developmental disorders characterized by cardiac, craniofacial and musculoskeletal abnormalities (Aoki et al., 2005; Cirstea et al., 2010; Niihori et al., 2006; Schubbert et al., 2006). However, the mechanism by which mutated Ras leads to developmental defects is largely unknown. Conversely, functions of normal Ras proteins in vivo have also been studied using knockout mice for the Ras genes. These studies have demonstrated that Kras-null mice die during embryonic development (Johnson et al., 1997; Koera et al., 1997), whereas mice deficient for both Hras and Nras grow normally (Esteban et al., 2001); this suggests that Kras might play a unique and indispensable role in development. However, it has also been reported that homozygous disruption of the Nras gene in combination with heterogeneous disruption of the Kras gene also causes embryonic death (Johnson et al., 1997). Moreover, knock-in mice expressing Hras instead of the endogenous Kras are viable (Potenza et al., 2005), and a transgenic allele harbouring the human, wild-type HRAS gene can rescue mice lacking three Ras genes from lethality (Nakamura et al., 2008), suggesting that endogenous Hras and Nras can partially compensate for loss of Kras. Furthermore, Ras single and compound knockout mice exhibit complex, lethal phenotypes that include hematopoietic, erythropoietic, liver, cardiac and neuronal abnormalities (Johnson et al., 1997; Khalaf et al., 2005; Koera et al., 1997; Matsu et al., 2002; Nakamura et al., 2008). These findings, together with the reported wide-spread expression of the three Ras genes (Nakamura et al., 2008), have made identifying the precise roles of the three Ras proteins in normal development elusive.

Differentiation, growth and maturation of the blood and lymphatic vasculature are orchestrated by many molecular and cellular processes (Adams and Alitalo, 2007). Ras is believed to serve as an essential component of intracellular signalling downstream from endothelial receptor tyrosine kinases. Previous studies using knockout mice have revealed that the regulators for Ras GTPases and Ras effectors – Nf1, p120 Ras-GAP (also known as Rasal1), Rasip1 and Braf – play important roles in normal blood vessel development (Henkemeyer et al., 1995; Wojnowski et al., 1997; Wu et al., 2006; Xu et al., 2009). In addition, mice homozygous for a mutated Pi3kea gene encoding the catalytic
The p110β isoform of PI3 kinase that does not interact with Ras exhibits lymphatic vessel hypoplasia (Gupta et al., 2007). These studies suggest that Ras might participate in the blood and lymphatic vascular development. More recently, it has been demonstrated in vitro that Hras-mediated VEGFA/VEGFR2 signalling promotes endothelial cell specification in mouse embryonic stem cell-derived vascular progenitor cells (Kawasaki et al., 2008), and that Hras regulates neovascularization and vascular permeability by activating distinct effectors in blood endothelial cells (BECs) in vitro and in vivo (Serban et al., 2008). Consistent with the above findings, it has been found that Hras knockout mice exhibit blood vessel malformation; however, this defect is transient (Kawasaki et al., 2008), and additional endothelial cell phenotypes in mice harbouring Ras mutations have not been reported, indicating that further studies are required to determine the precise functions of Ras in angiogenesis and lymphangiogenesis.

In the present study, we focus on a lymphatic vascular phenotype we unexpectedly identified through a phenotypic characterization of Ras compound knockout mice (Nakamura et al., 2008). Using genetically modified mice, we found that compound disruption of the three Ras genes and overexpression of normal Hras protein in an endothelial cell lineage led to lymphatic vessel hypoplasia and hyperplasia, respectively. Using mouse lymphatic endothelial cells (LECs) in vitro, we also show how overexpressed Ras or knockdown of Ras in LECs lead to aberrant lymphatic vessel growth at the cellular and molecular levels. Finally, we demonstrate for the first time that the three types of Ras proteins cooperatively and dose-dependently regulate lymphatic vessel growth by modulating VEGFR3 signalling in LECs.

MATERIALS AND METHODS

**Mice**

We purchased C57BL/6J and MCH:ICR mice from CLEA Japan (Tokyo, Japan); Tie2(Tek)-Cre transgenic (Koni et al., 2001), ROSA26-loxP-stop-loxP-β-geo knock-in (Mao et al., 1999) and ROSA26-mT/mG knock-in mice (Muzumdar et al., 2007) from the Jackson Laboratory (Bar Harbor, ME, USA), and FLP66 transgenic mice (Takeuchi et al., 2002) from RIKEN BRC (Muzumdar et al., 2007). Ras knockout mice (the sequence was verified. The first time that the three types of Ras proteins cooperatively and dose-dependently regulate lymphatic vessel growth by modulating VEGFR3 signalling in LECs.

**Generation of Vegfr3-Cre transgenic mice**

An approximately 7 kb PacI-NotI fragment harbouring the mouse Vegfr3 promoter region was subcloned from the RPCI23-118J11 BAC clone (Invitrogen). The Vegfr3 promoter and a nlsCre CDNA (a gift from Dr K. Rajewsky, Harvard Medical School, MA, USA) followed by a poly-adenylation (pa) signal sequence from the mouse Pkg gene were assembled into a cloning vector. The SalI-digested insert fragment was gel-purified and dissolved in PBS before microinjecting into the pronuclei of fertilized eggs from C57BL/6J mice. Injected eggs were transferred into the oviduct of pseudo-pregnant MCH:ICR female mice. Founder mice were identified by PCR genotyping and Cre activity was initially assessed by crossing with ROSA26-loxP-stop-loxP-β-geo knock-in mice (Mao et al., 1999). Cre activity in lymphatic endothelial cells (LECs) in one line (No. 44) was verified by crossing with ROSA26-mT/mG knock-in mice (Muzumdar et al., 2007).

**Generation of Vegfr3-EGFP knock-in mice**

A PacI-NotI fragment of the Vegfr3-Cre transgene was used as a 5′ homology arm of the targeting vector, and a 1-2 kb 3′ homology arm harbouring an intronic region between exon 1 and 2 of Vegfr3 was PCR-amplified using a primer set (forward, 5′-GCGGTGTGAACGTCTTCCGGGTTC-3′; and reverse, 5′-GAGTTGACGGCCAGGGAAGTC-3′) and the RPCI23-118J11 BAC DNA. The fragment was cloned downstream of the 5′ arm and the sequence was verified. An EGFP cDNA with a Pkg pa signal sequence followed by an FRT-flanked Pkg-gb2-neo cassette (Ichise et al., 2009) was ligated into the NotI site, upstream of the first ATG of the Vegfr3 gene. The resulting fragment was cloned into pUC-DT-A (a gift from Dr Takeshi Yagi, Osaka University, Japan) (Yagi et al., 1993). A C57BL/6J × 129/SvEv F1 male ESC line was established and used for gene targeting. Pack-digested, linearized vector (20 μg) was introduced into ESCs. Mice were generated using two independent, correctly targeted clones as assessed by PCR genotyping of the ESCs. Heterozygous Vegfr3-EGFP knock-in mice without the neomycin-resistance gene were generated by crossing to the FLP-deleter strain FLP66 (Takeuchi et al., 2002). The mice were then backcrossed at least six times to C57BL/6J mice before being used for this study.

**Immunohistochemistry and immunocytochemistry**

Immunostaining of sections, wholemount tissues and cells was performed as previously described (Ichise et al., 2009; Yamaguchi et al., 2008). The following primary antibodies were used: rat anti-mouse Lyve1 (R&D Systems, Minneapolis, MN, USA); rat anti-mouse CD31 (BD-Pharmingen, Franklin Lakes, NJ, USA); rat anti-EGFP (Nacalai-Tesque, Kyoto, Japan); biotinylated rat anti-mouse CD31 (BD-Pharmingen); biotinylated goat anti-mouse Lyve1 (R&D Systems); and rabbit anti-SV40 T antigen (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A streptavidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) was used with biotinylated antibodies. The Histofine reagent (Nichirei Biosciences, Tokyo, Japan), streptavidin-HRP (NEN/Perkin-Elmer, Waltham, MA, USA), and the TSA/ TSA PLUS HRP Detection System (NEN/Perkin-Elmer) were used for detection. Fluorescence micrographs were acquired with Olympus microscopes equipped with digital cameras (IX70/DP70, SXZ12/DP72, MVX-CSU; Olympus, Tokyo, Japan) or a BioRevo 9000 microscope (Keyence, Osaka, Japan). Micrographs in figures are representative of two independently stained specimens from three or more mice. We verified that the auto-fluorescent EGFP signal, or false-positive staining by normal IgG or isotype control antibodies, did not affect the staining results.

**Preparation and culture of mouse LECs**

Conditionally immortalized mouse mesenteric LECs were prepared and maintained as described previously (Yamaguchi et al., 2008). Wild-type and Hras-overexpressing LECs were obtained from mesenteries of 10- to 20-
day-old T26/Tie2-Cre double transgenic mice and T26/Vegfr3-Cre/CGH triple transgenic mice, respectively. The LECs were cultured in EGM-2MV medium (Lonza, Basel, Switzerland) on gelatinized culture dishes at 33°C with 5% CO2 in a humidified incubator. For Fig. 3A, 5×10^4 cells were placed on gelatinized 6 cm dishes and cultured in EGM-2MV medium (Lonza) at 33°C for 6 hours. Trypsinized cells were counted every 2 days for 6 days and the cell numbers of three independent dishes were analyzed. In Fig. 3 and Fig. 4, we used the Cell Proliferation Reagent WST-1 and the Cell Death Detection ELISA PLUS Kit (Roche Diagnostics K. K., Tokyo, Japan) according to the manufacturer’s instructions. For Fig. 5, Fig. 6 and Fig. S5 in the supplementary material, MAZ51 and FTI-277 (Calbiochem/Merck, Darmstadt, Germany) were dissolved in DMSO, diluted in PBS and used for cell culture. PBS containing only DMSO was used for control experiments. After overnight starvation, as described above, cells were cultured in the presence of MAZ51 at 33°C for 4 hours. In Fig. 6, after the 4-hour incubation in the presence of MAZ51, rat VEGFC (R&D Systems) was added to the medium at a final concentration of 100 ng/ml, and then cells were incubated at 33°C for 10 minutes before harvesting them for analysis. In Fig. 5 and Fig. S5 in the supplementary material, cells were cultured in EGM-2MV medium (Lonza) containing 10 μM FTI-277 at 33°C for 2 days (Fig. 5B; see also Fig. SSB-D in the supplementary material) or 3 days (see Fig. SSA in the supplementary material). The pre-treated cells were cultured in EBM-2 supplemented with 0.5% foetal calf serum (FCS) and FTI-277 overnight in gelatinized dishes prior to stimulation with VEGFA or C, or the EGM-2MV medium on gelatinized culture dishes at 33°C for 1 day, then 1 μg/ml Dil (Molecular Probes/Invitrogen) was added to the medium. The cells were incubated at 37°C for 30 minutes, washed twice in PBS and then trypsinized. The Dil-labelled cells were counted, seeded on the Matrigel-coated dishes at a cell concentration of 7×10^4 cells per well, then cultured in EBM-2 (Lonza) supplemented with 0.5% FCS at 33°C for 24 hours in the presence of or absence of inhibitors. For Fig. 4E, cells on the Matrigel-coated dishes were cultured in EGM-2MV medium (Lonza) at 33°C for 24 hours. The 1 cm2 areas covered by Dil-labelled cellular structures were measured using a BioRevo-9000 imaging system (Keyence, Osaka, Japan). Three independent wells were used for each analysis.

Network formation of LECs on Matrigel

12-well Matrigel (BD Pharmingen)-coated dishes (9.5 mg/ml; 100-200 μl per well) were used to analyze network formation of tsASBT-expressing endothelial cells. Conditionally immortalized LECs were cultured in EGM-2MV medium on gelatinized culture dishes at 33°C for 1 day, then 1 μg/ml Dil (Molecular Probes/Invitrogen) was added to the medium. The cells were incubated at 37°C for 30 minutes, washed twice in PBS and then trypsinized. The Dil-labelled cells were counted, seeded on the Matrigel-coated dishes at a cell concentration of 7×10^4 cells per well, then cultured in EBM-2 (Lonza) supplemented with 0.5% FCS at 33°C for 24 hours in the presence or absence of inhibitors. For Fig. 4E, cells on the Matrigel-coated dishes were cultured in EGM-2MV medium (Lonza) at 33°C for 24 hours. The 1 cm2 areas covered by Dil-labelled cellular structures were measured using a BioRevo-9000 imaging system (Keyence, Osaka, Japan). Three independent wells were used for each analysis.

Western blot analysis and ELISA

Cell lysates (20 μg) were resolved by SDS-PAGE, and semi-dry-blotted onto PVDF membranes (Millipore, Billerica, MA, USA). Western blot analysis was performed using the following primary antibodies: mouse anti-Pan-Ras (Calbiochem/Merck); rat anti-VEGFR3 (Bioscience, San Diego, CA, USA); rabbit anti-Prox-1 (Upstate/Millipore, Billerica, MA, USA); rabbit anti-tubulin, rabbit anti-phosphorylated p42/44 MAPK, rabbit anti-p42/44 MAPK, rabbit anti-phosphorylated Akt and anti-Akt (Cell Signaling Technology, Danvers, MA, USA); and rabbit anti-Sos1 (Santa Cruz Biotechnology). The secondary antibodies were: swine anti-goat IgG HRP, goat anti-rabbit IgG HRP anti-membrane IgG HRP and goat anti-rat IgG HRP (Biosource/Invitrogen, Carlsbad, CA, USA). Enhanced chemiluminescence (ECL; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and X-ray film (Fujiﬁlm, Tokyo, Japan) were used for detection. Results shown in the ﬁgures represent triplicate experiments. Western blot images were analyzed using the NIH Image J software. When detecting GTP-bound Ras, a Ras activation assay kit (Upstate/Millipore) was used according to the manufacturer’s instructions. To determine if they had white fluid in the abdomen. Tail tips (1-2 mm) of newborns were checked at least once a day for 5 days after birth to identify the affected mice.

Quantitative real-time RT-PCR

Total RNA of ECs and adult tissues were obtained using TRIzol reagent (Invitrogen), treated with DNase I and ﬁltered. First-strand cDNA was synthesized using SuperScript III First Strand Synthesis System (Invitrogen), cDNA corresponding to 20 ng of total RNA was used for PCR under the following amplification conditions: 94°C for 2 minutes, 30 cycles (35 cycles for KrasA) of 94°C, 60°C (55°C for KrasA) and 72°C for 1 minute each, with a ﬁnal extension at 72°C for 5 minutes. PCR products were veriﬁed by electrophoresis on 3% agarose gels in TBE buffer. Primers used for this study are as follows: 5’-GACACGGAACAGCCTGACAGTGTTAGCAAGG-3’ and 5’-TACATAACTGTCACCTGTGTCTCT-3’ for KrasA and KrasB; 5’-GACACGGAACAGCCTGACAGTGTTAGCAAGG-3’ and 5’-TTACATTTAACAAGCTTATTTATAT-3’ for KrasA; 5’-GAGTCTCGGAGCGCCAGCTTGCCTGC-3’ and 5’-TCATGGACAGCACAACATTGCGACGT-3’ for Hras; 5’-GACACAAAGCAGGCCACAGGAACCATCGGTGAC-3’ and 5’-CTCAGATGGTGACAGGCGCA-3’. The primers for this study are as follows: 5’-CACTCATACGACAGGCGCAACC-3’ and 5’-AGGAGTAGCCCTTCCTCAGGGTC-3’. For the assessment of amount of each of the three Ras transcripts, amplicons formed via PCR using a primer set (5’-CAGAAACACTTGTTGGA3’ and 5’-TTATGTCGCCAAATACACA-3’, 1, dITP) were digested by DpnII and PvuII and then resolved by electrophoresis on 4% agarose gels in TBE buffer.

We conﬁrmed that no ampliﬁcation was produced using reverse transcriptase-minus RNA and the primer sets described above during RT-PCR analysis.

Scoring of lymphatic vessel growth in the small intestine

The small intestines of newborns at postnatal day 2 (P2; the day of birth is designated P0) were dissected and divided into eight segments of equal length. The EGFP ﬂuorescence of EGFP-expressing lymphatic vessels in each intestinal segment from Vegf3EGFP knock-in mice was observed using"
an Olympus fluorescence microscope equipped with a CCD camera (SZX12/DP72; Olympus) and was scored by assessment of the relative area of EGFP-expressing lymphatic vessels as follows: 0, absent; 1, up to 25%; 2, up to 50%; 3, up to 75%; and 4, up to 100% of the length of the segment. PCR genotyping for the Ras genes was conducted after scoring. The sums of the scores of all eight segments (maximum 32) were analyzed.

Statistics
Comparisons in this study were made using two-tailed paired Student t-tests (α=0.05), and the comparisons in Fig. 7 were made with Welch’s correction.

RESULTS
Lymphatic vessel hypoplasia is a phenotype commonly observed in newborn ras compound knockout mice
Through a phenotypic characterization of Ras compound knockout mice (Nakamura et al., 2008), we found chylous ascites, which is a typical symptom of lymphatic vessel abnormalities (Karpanen and Alitalo, 2008), and is a common phenotype in Ras compound knockout newborns (Fig. 1A,B). Double immunostaining for lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1) (Prevo et al., 2001) and CD31 showed that, in contrast to blood vessels, lymphatic vessels had developed abnormally in large parts of the small intestines of Ras compound knockout newborns (Fig. 1C for Nras<sup>+/−</sup>/Kras<sup>+/−</sup> newborns; data not shown for others). The transgenic allele rasH2, harbouring the human HRAS genomic region (Saitoh et al., 1990), rescued Ras compound knockout newborns from chylous ascites (Fig. 1D) as well as other lethal phenotypes (Nakamura et al., 2008). Total Ras protein levels in the small intestine decreased in line with decrease of wild-type Ras alleles, and recovered by overexpression of Hras from the rasH2 allele (Fig. 1E). These results suggest that the three Ras genes have overlapping functions in cells contributing to lymphatic vessel development; however, specifying the exact cell type is difficult because the Ras genes are expressed ubiquitously (Nakamura et al., 2008).

Overexpression of Hras in an endothelial cell lineage leads to lymphatic vessel hyperplasia in mice
In order to determine the cell types contributing to these abnormalities, we generated transgenic mice expressing wild-type Hras in a specific cell lineage using a Cre/loxP recombination-driven expression system (see Fig. S1A in the supplementary material; hereafter referred to as CGH mice). Initially, we investigated whether endothelial expression of Hras could rescue
Ras compound knockout mice from the lymphatic vascular phenotypes and we found that endothelial cell-specific expression was achieved using Tie2-Cre transgenic mice (Koni et al., 2001) (see Fig. S1B in the supplementary material). However, unexpectedly, endothelial overexpression of normal Hras resulted in severe edema and perinatal death (Fig. 2A,B). Blood vascular development, such as arterial and venous development, was unaffected (see Fig. S1B in the supplementary material) and blood vessels in peripheral tissues appeared to be normal (Fig. 2C for the skin; data not shown for others); however, dilated and hyperplastic lymphatic vessels were observed in the skin of the double-transgenic embryos (Fig. 2C). As blood vessel dysfunction might affect normal lymphangiogenesis, instead of the Tie2-Cre transgenic mice, we also used a Vegfr3-Cre transgenic mouse line that drives recombination in a subpopulation of LECs during postnatal life (see Fig. S2A in the supplementary material), rarely in LECs at birth (data not shown) and never in BECs (see Fig. S2A in the supplementary material). Approximately 60% of central lacteals were EGFP-positive in the small intestine of the Cre reporter/Vegfr3-Cre double-transgenic adult mice, as assessed by immunostaining (data not shown). CGH/Vegfr3-Cre double-transgenic mice, in which a subset of lymphatic vessels was EGFP-positive as expected (see Fig. S3C in the supplementary material), also showed lymphatic hyperplasia consisting of EGFP-positive lymphatic vessels in the heart and mesentery (Fig. 2D; see also Fig. S2B in the supplementary material) and chylothorax (Fig. 2E), suggesting that overexpressed Ras in CGH/Tie2-Cre LECs induced lymphatic vessel hyperplasia and dysfunction.

Overexpressed Hras confers increased cell viability, anti-apoptosis and sustained MAPK activation in LECs under serum-depleted culture conditions and stimulates endothelial sheet formation by LECs on Matrigel in vitro

To examine how Ras influences hyperplasia in LECs, we isolated conditionally immortalized LECs from transgenic mice expressing the weak-acting, mutated SV40 large T antigen (Yamaguchi et al., 2008). Lymphatic endothelial markers VEGFR3 (also known as Flt4) (Kaipainen et al., 1995) and Prospero-related homeobox protein 1 (Prox1) (Wigle and Oliver, 1999) were expressed in both genotypes of LECs, and also confirmed that Hras was overexpressed in T26/Vegfr3-Cre triple-transgenic LECs as expected (see Fig. S3C in the supplementary material). Total Ras protein levels in Hras-overexpressing LECs are estimated to be at least five times greater than that of control LECs (see Fig. S3D in the supplementary material). Consistent with our results suggesting that all three Ras genes contribute to lymphatic vessel growth (Fig. 1), transcripts for all three Ras genes were found in LECs by RT-PCR (see Fig. S4A in the supplementary material). Regarding two Kras splice variants, levels of KrasB transcripts were higher than those of KrasA, which is the tissue-specific transcript encoding a distinct Kras isoform dispensable for normal mouse development (Pells et al., 1997; Plowman et al., 2003). In addition, restriction digest analysis of RT-PCR products amplified using a common
Hras activation is required for sustained MAPK activation and endothelial sheet formation by Hras-overexpressing LECs

To further examine whether MAPK activation and endothelial sheet formation depend on Ras activation, we performed similar experiments in the presence of a farnesyl transferase inhibitor, FTI-277, whose target is farnesylation of Hras because N- and Kras are geranylgeranylated alternatively in the presence of FTI (Whyte et al., 1997). The amount of GTP-bound Ras decreased in LECs cultured in the presence of FTI for three days (see Fig. S5A in the supplementary material) and FTI inhibited hyper-activation of MAPKs in Hras-overexpressing LECs (see Fig. S5B,C in the supplementary material). In addition, sheet formation of Hras-overexpressing LECs was also inhibited by FTI treatment (see Fig. S5A in the supplementary material). Taken together, these results suggest that overexpressed Hras becomes farnesylated and GTP-loaded in response to upstream signals, which then leads to MAPK activation and enhanced endothelial sheet formation. As no mutation was found in the Hras gene, followed by Hras, KrasB and then KrasA (see Fig. S4C in the supplementary material). Next, we compared their proliferation and viability characteristics. There were no significant differences between control and Hras-overexpressing LECs cultured in normal medium (Fig. 3A,B). However, most strikingly, Hras-overexpressing LECs were viable and anti-apoptotic in serum-depleted medium (Fig. 3B,C). We next examined intracellular signalling in LECs. GTP-bound Ras in wild-type LECs, which remained at low levels in serum-depleted cultures, increased after stimulation by VEGFA, a ligand for VEGFR2, or VEGFC, a ligand for VEGFR2 and VEGFR3 (Joukov et al., 1996) (Fig. 3D). By contrast, GTP-bound Ras in Hras-overexpressing LECs was abundant in serum-depleted culture cells (Fig. 3D). Similarly, MAPK activation in both types of LECs was significantly induced by VEGFA or C, but MAPKs in Hras-overexpressing LECs remained activated in serum-depleted cell cultures (Fig. 3D). In addition, Akt was activated at similar levels in the two types of LECs (Fig. 3D). We also examined two-dimensional endothelial cell network formation on Matrigel. Intriguingly, Hras-overexpressing LECs formed island-like sheet structures in grids of endothelial networks in serum-depleted medium; however, these sheets were not formed by wild-type LECs (Fig. 3E). Starved Hras-overexpressing LECs on Matrigel were also significantly more viable than wild-type LECs (Fig. 3F). These findings suggest that under growth factor-depleted conditions, overexpressed Hras stimulates lymphatic endothelial viability and leads to lymphatic vessel hyperplasia by elevating MAPK activation in LECs.

Knockdown of Ras proteins in LECs also affects VEGFR3 protein expression

We performed Ras knockdown experiments using control LECs to examine whether downregulation of Ras proteins also affects the LEC phenotype. In contrast to the LEC characteristics induced by primer set for Ras genes (see Fig. S4B in the supplementary material) revealed that the most abundantly expressed Ras gene was Nras, followed by Hras, KrasB and then KrasA (see Fig. S4C in the supplementary material).
the overexpression of Hras described above, we found that knockdown of Ras proteins by siRNAs (Fig. 4A) reduced LEC viability (Fig. 4B) but did not induce apoptosis (Fig. 4C) in a normal culture condition, suggesting that Ras knockdown LECs proliferate slower than control LECs. Additionally, knockdown of Ras impaired responses to VEGF signalling as assessed by MAPK activation (Fig. 4D). In particular, mouse VEGF-D, stimulating only mouse VEGFR3 in contrast to VEGFC and human VEGF-D, which stimulate both VEGFR2 and VEGFR3 (Baldwin et al., 2001), activated MAPKs in control LECs; however, this activation was impaired in Ras knockdown LECs (Fig. 4D), suggesting that Ras signalling correlates with VEGFR3. Knockdown of Ras also impaired the ability of LECs to migrate (Fig. 4E) and suppressed endothelial networks by LECs on Matrigel (Fig. 4F). Unexpectedly, Western blotting revealed that VEGF3 protein expression was significantly reduced by Ras knockdown (Fig. 4G). These results suggest that Ras proteins promote VEGFR2- and VEGFR3-mediated MAPK signalling and also maintain VEGFR3 expression levels in LECs.

VEGFR3 protein expression level is modulated transcriptionally by Ras signalling

To determine whether VEGFR3 protein levels correlate with Ras protein levels, we quantitatively examined VEGFR3 in Hras-overexpressing LECs and found higher levels of VEGFR3 when Ras was overexpressed (Fig. 5A). Ras activation is required for VEGFR3 expression because VEGFR3 protein levels were reduced by FTI treatment (Fig. 5B). Together with the result that Vegfr3 transcript levels were elevated in Hras-overexpressing LECs and were reduced in Ras knockdown LECs (Fig. 5C), these data suggest that Ras signalling modulates the transcription of the Vegfr3 gene in LECs.

Enhanced endothelial sheet formation by Hras-overexpressing LECs is dependent on VEGFR3 signalling

To determine whether the Hras-induced VEGFR3 increase has a functional effect on LECs, we performed a network formation assay in the presence of MAZ51, a selective kinase inhibitor of VEGFR3.
Blockage of VEGFC-induced phosphorylation of VEGFR3 in LECs was observed in the presence of 5 μM of MAZ51 in western blots (Fig. 6A) and lower concentrations of MAZ51 could sufficiently inhibit endothelial network formation in both types of LECs in a dose-dependent manner (Fig. 6B), suggesting that the increase of VEGFR3 by Hras overexpression might be involved in the hyperplasic phenotype of Hras-overexpressing LECs in vitro.

The Ras genes modify VEGFR3-dependent lymphatic vessel growth in a gene dose-dependent manner

Finally, we investigated whether the Ras genes interact genetically with the Vegfr3 genes. Heterozygous mutant mice for the Vegfr3 gene show similar phenotypes of Ras compound knockout mice (Karkkainen et al., 2000; Karkkainen et al., 2001) and a subpopulation of heterozygous-null mutants for Vegfr3 develop chylous ascites (Haiko et al., 2008). We generated Vegfr3-EGFP knock-in mice (see Fig. S6 in the supplementary material) and confirmed that a subpopulation of the mice developed chylous ascites (Fig. 7A) and intestinal lymphedema due to lymphatic vessel hypoplasia (Fig. 7B). By contrast, in the presence of the rasH2 transgenic allele, either Kras+/+Vegfr3−/−, or Kras−/−Vegfr3+/− newborns scarcely showed chylous ascites (Fig. 7A) or lymphatic vessel hypoplasia (data not shown). In order to assess lymphatic vessel development, we scored GFP-positive lymphatic vessels in the small intestine (Fig. 7B,C). Together with the observation of chylous ascites, the scoring clearly indicated that the deficiency in the Kras and Vegfr3 genes synergistically caused lymphatic vessel hypoplasia in the small intestine, and also that the HRAS transgene promoted lymphatic vessel growth counteracting the haploinsufficiency effect of the Vegfr3 gene.

DISCUSSION

Taken together, our results strongly suggest that Ras in LECs acts as a signalling switch directing expression of VEGFR3, as well as a signalling switch downstream of VEGFR3, and that three types of Ras proteins cooperatively play important roles in cell viability, signal transduction, migration and network formation in vitro and lymphatic vessel growth in vivo by modulating VEGFR3 signalling in LECs (Fig. 8).
Three Ras gene products synergistically promote normal lymphatic vessel growth, and the increase and decrease of total Ras proteins causes lymphatic vessel hyperplasia and hypoplasia, respectively.

Kras is the most influential among the Ras genes in lymphatic vessel development, as suggested by the result using Ras compound knockout mice; however, its transcription levels are lower than those of Nras and Hras. The three Ras proteins can compensate for each other as shown in this study and previous studies, but it is likely that Kras might have different characteristics in signalling probably owing to its post-translational modification and trafficking in LECs, as suggested by previous biochemical studies (Karnoub and Weinberg, 2008).

It seems probable that Ras compound knockout embryos dying in utero would exhibit impaired blood vascular development owing to inappropriate Ras signalling in BECs (Kawasaki et al., 2008; Serban et al., 2008); however, our results showed no overt blood vascular phenotypes. We found in CGH/Tie2-Cre embryos that

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**Fig. 7.** The Ras genes modify VEGFR3-dependent lymphatic vessel growth in a gene dose-dependent manner. (A) Frequency of chylous ascites observed in newborns carrying a Vegfr3EGFP knock-in allele in the presence or absence of a Kras-null allele and/or a rasH2 transgenic allele. (B) EGFP-expressing lymphatic vessels (labelled in greyscale images of lower panels) in the small intestine of newborns carrying a Vegfr3EGFP allele. Asterisks indicate a lymphedematous part of the small intestine lacking GFP-expressing lymphatic vessels. Scale bar: 500 μm. (C) Lymphatic vessel development in each newborn was scored by assessment of the area covered by GFP-labelled lymphatic vessels in eight equal segments of the small intestine: 0, absent; 1, up to 25%; 2, up to 50%; 3, up to 75%; 4, up to 100%. The sums of the scores for each newborn (postnatal day 2) were compared. Error bars, s.d. **, \( P < 0.00001 \) (versus Vegfr3EGFPKras+/+ mice); *, \( P < 0.05 \) (versus Vegfr3EGFPKras+/+ mice); #, \( P < 0.000001 \) (versus Vegfr3EGFPKras+/– mice).

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**Fig. 8.** Schematic representation of the role of Ras in lymphangiogenesis. Ras protein expression levels in LECs not only influence GTP-bound Ras levels and MAPK activity levels as regulated by upstream signals via VEGFR2, VEGFR3 and other kinases, but also VEGFR3 expression levels. The increase or decrease in VEGFR3 expression modulated by Ras/MAPK signalling causes lymphatic vessel hyperplasia or hypoplasia, respectively, by changing lymphatic endothelial cell characteristics in terms of viability, proliferation, anti-apoptosis, network formation and migration.
overexpression of Hras affected lymphatic vessel growth but not blood vessel growth, and that LEC characteristics are highly dependent on both Ras function and Ras protein expression levels. LECs arise from a subset of venous BECs and then migrate away from blood vessels to peripheral tissue (Adams and Alitalo, 2007), where there is a reduced supply of nutrients and oxygen. The LEC migration requires VEGFC, which is expressed predominantly by mesenchymal cells in peripheral tissue (Karkkainen et al., 2004). Ras proteins might help LECs survive in a serum-depleted mesenchymal environment and respond to VEGFC by sustaining MAPK activity and upregulating VEGFR3 expression during lymphatic vessel development.

Ras and VEGFR3 expression levels and MAPK activity levels, correlate with each other. However, it remains unclear how VEGFR3 expression levels are regulated by Ras/MAPK signalling or what upstream signals activate Ras in terms of VEGFR3 expression. It also remains unknown whether Ras/MAPK signalling is the only pathway downstream from VEGFR3 responsible for VEGFR3-dependent lymphatic vessel growth. E-twenty six (Ets) transcription factors, which are nuclear effectors of Ras/MAPK signalling (Wasylyk et al., 1998), are potential candidates for Ras/MAPK-dependent transcriptional regulators of the Vegfr3 gene. Intriguingly, mice harbouring a homozygous mutation in an Ets family gene develop a lymphatic vascular phenotype (Ayadi et al., 2001). It would be important to address the issue concerning Vegfr3 transcriptional regulation by the Ras/MAPK/Ets signalling pathway in future studies.

Regarding VEGFR3/Ras/MAPK signalling, a previous study reported that mouse embryos deficient for the Sprd1 and Sprd2 genes, which encode proteins that negatively regulate growth factor-induced MAPK activation, are embryonic lethal and show abnormal lymphatic vessels. This study also suggested that inhibition of VEGF/VEGFR3 signalling by Sprd proteins might be important for lymphangiogenesis (Taniguchi et al., 2007), which is consistent with the idea that Ras/MAPK signalling is the major pathway downstream from VEGFR3. However, impaired lymphatic vessel growth was also observed in mice homozygous for the mutated Ptd3ca gene encoding the PI3 kinase p110α isoform, which lacks the Ras-binding domain (Gupta et al., 2007). Although Akt activation was not significantly affected in Ras-overexpressing or knockdown LECs in this study, it is possible that the Ras/PI3 kinase pathway might participate in VEGFR3 signalling in LECs. In a future study, we plan to address the relationship between these pathways using amino acid substitution mutants for Ras and VEGFR3, leading to activate a limited signalling pathway.

VEGFR3 heterozygous mis-sense mutations have been found in individuals with hereditary lymphedema (Karkkainen et al., 2000) and Chy (Flt4-β) mice showing chylous ascites and lymphoedema (Karkkainen et al., 2001). In a previous study (Haiko et al., 2008), and in this report, genetically engineered Vegfr3 heterozygous-mutant mice showed chylous ascites and lymphoedema. However, these mutations showed incomplete penetrance of the phenotypes, suggesting that modifier genes might be involved in lymphatic vessel phenotypes caused primarily by dysfunction of VEGFR3. As demonstrated in this study, polymorphisms or mutations in the Ras genes and other genes encoding proteins that participate in Ras/MAPK signalling are probable candidates. Furthermore, lymphoedema, lymphangiomatosis/hygroma and chylothorax have been reported in Noonan and cardio-facio-cutaneous syndromes caused by germline mutations in KRAS and other genes that encode proteins involved in the Ras/MAPK signalling pathway (Chan et al., 2002; Donnenfeld et al., 1991; Evans et al., 1991; Witt et al., 1987). Our mouse models that overexpress Hras using an artificial gene construct might not sufficiently model human diseases where physiological upregulation of Ras or expression of weak-activating mutant forms of Ras proteins occur. However, Ras-induced lymphatic vessel hyperplasia might be a potential model for lymphangioma to explore therapeutic treatments for humans, especially when caused by Ras/MAPK signalling defects.

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Competing interests statement

The authors declare no competing financial interests.

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