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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. Overexpression of normal Ras in lymphatic endothelial cells leads to sustained MAPK activation, cellular viability and enhanced endothelial network formation under serum-depleted culture conditions in vitro, and knockdown of endogenous Ras in lymphatic endothelial cells impairs cell proliferation, MAPK activation, cell migration and endothelial network formation. Ras overexpression and knockdown result in up- and downregulation of vascular endothelial growth factor receptor (VEGFR) 3 expression, respectively, in lymphatic endothelial cells in vitro. The close link between Ras and VEGFR3 in vitro is consistent with the result that Ras knockout and transgenic alleles are genetic modifiers in lymphatic vessel hypoplasia caused by *Vegfr3* haploinsufficiency. Our findings demonstrate a cooperative function of the three Ras proteins in normal development, and also provide a novel aspect of VEGFR3 signalling modulated by Ras in lymphangiogenesis.

KEY WORDS: Ras, Endothelial cell, Lymphangiogenesis, Mouse

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; Matsui 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 Rasa1), 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 *Pi3kca* gene encoding the catalytic

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p110α isoform of PI3 kinase that does not interact with Ras exhibit 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 (Tsukuba, Japan). CAG-β-geo-tsA58T Ag transgenic mice (referred to as T26 transgenic mice in this study) were described previously (Yamaguchi et al., 2008). *Hras*, *Nras*, and *Kras* knockout mice (Ise et al., 2000; Koera et al., 1997; Nakamura et al., 2008) and *rasH2* transgenic mice (Saitoh et al., 1990) were kindly provided by Dr Motoya Katsuki (Inter-University Research Institute, National Institutes of Natural Sciences, Japan). Ras knockout mice and *rasH2* transgenic mice in the B6/129/DBA2 mixed genetic background (Fig. 1A,D) or B6 genetic background (backcrossed to C57BL/6J for more than 20 generations; all figures other than Fig. 1A,D) were used for this study. Generation of new genetically engineered mouse strains is described below. All mice were housed under pathogen-free conditions. All mouse work conformed to guidelines approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

Generation of CAG-β-geo-*Hras* IRES EGFP (CGH) transgenic mice

The cDNA encoding mouse Hras was PCR-amplified from mouse brain cDNA, cloned into pIRES2-EGFP (Clontech/TAKARA, Shiga, Japan), and the sequence was verified. The *Hras*-IRES-EGFP cDNA was subcloned into the *Swa*I site of pCGX (Niwa et al., 1991; Yamaguchi et al., 2008). A transgene cassette from *Sal*I-digested pCGX harbouring the *Hras*-IRES-EGFP cDNA was gel-purified and dissolved in PBS. The transgene (10 μg) was introduced into E14.1 embryonic stem cells (ESCs) by electroporation. Transgene-expressing ESCs were selected by culturing in medium containing 400 μg/ml G418 (Invitrogen) for 7 days. G418-resistant colonies were picked and expanded for PCR genotyping and the formation of embryoid bodies (EBs). Of the 48 G418-resistant ESC clones isolated, several clones were analyzed for the presence of the *Hras*-EGFP cDNA and for widespread expression of β-geo in EBs. *Cre*-mediated EGFP expression in the clones was verified. For the production of transgenic mice, ESCs were injected into B6 blastocysts, which were transplanted into the uteri of pseudo-pregnant MCH:ICR female mice. Chimeric male mice were then crossed with B6 female mice. CGH transgenic mice were back-crossed to B6 mice at least six times then used for analysis. For genotyping, PCR and/or X-gal staining of tail tips was performed.

Generation of *Vegfr3-Cre* transgenic mice

An approximately 7 kb *Pac*I-*Not*I 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 *Pgk* gene were assembled into a cloning vector. The *Sal*I-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 *Pac*I-*Not*I 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′-GCGTTTAAACGATCCTTC-GGGTTC-3′ and reverse, 5′-GAGTCGACGGCCCAGGGAAGTC-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 *Pgk* pA signal sequence followed by an FRT-flanked *Pgk*-gb2-neo cassette (Ichise et al., 2009) was ligated into the *Not*I 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. *Pac*I-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-GFP (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, SZX12/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% CO_2 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 days. 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. S5B-D in the supplementary material) or 3 days (see Fig. S5A 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 cells were DiI-labelled and cultured in EBM-2 supplemented with 0.5% FCS and FTI-277 overnight on Matrigel, as described below.

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 tsA58T-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 DiI (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 DiI-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. 4F, cells on the Matrigel-coated dishes were cultured in EGM-2MV medium (Lonza) at 33°C for 24 hours. The 1 cm^2 areas covered by DiI-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 (eBioscience, San Diego, CA, USA); rabbit anti-Prox1 (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-hamster 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. ELISAs were performed using the PathScan MAPK ELISA Kit (Cell Signaling Technology) according to the manufacturer's instructions.

Growth factor stimulation

For analysis of cellular responses to growth factors, cells were cultured in EBM-2 basal medium (Lonza) supplemented with 0.5% FCS included in the EGM-2MV bullet kit at 33°C for 16 hours. Prior to stimulation with growth factors, the medium was replaced with fresh EBM-2/0.5% FCS. Recombinant human $VEGF_{165}$ (Peprotech EC, London, UK), rat VEGFC, or mouse VEGFD (R&D Systems) were added to the medium at final concentrations of 100 ng/ml. Cells were then incubated at 33°C for 10 minutes before harvesting them for analysis.

Knockdown experiments

Knockdown experiments were performed using mixed Stealth RNAi siRNA duplexes for three Ras genes, the corresponding scrambled Stealth RNAi siRNA controls and Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Prior to knockdown experiments, transfection was optimized by monitoring transfection efficiency of the BLOCK-iT Alexa Fluor Red Fluorescent Control (Invitrogen). Sense-strand sequences of the siRNA duplexes used in this study were: 5′-GGCAGCAUAAAUUGCGGAAACUGAA-3′ for *Hras*, 5′-GAACCACUUUGUGGAUGAAUAUGAU-3′ for *Nras* and 5′-GCGCCUUGACGAUACAGCUAAUUCA-3′ for *Kras*. Cells cultured for 72 hours after transfection were used for analyses. For the migration assay, an Oris Cell Migration Assay Kit (Platypus Technologies, Madison, WI, USA) was used according to the manufacturer's instructions. Cells at 72 hours post-transfection were DiI-labelled, fixed in 4% PFA, then measured using a BioRevo-9000 imaging analysis system (Keyence). More than three independent wells were used for each analysis.

RT-PCR

Total RNA from ECs and adult tissues were obtained using TRIzol reagent (Invitrogen), treated with DNase I and purified. 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 *Kras*A) of 94°C, 60°C (55°C for *Kras*A) 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′-GACACGAAACAGGCTCAGGAGTTAGCAAGG-3′ and 5′-TCACATAACTGTACACCTTGTCCT-3′ for *Kras*A and *Kras*B; 5′-GACACGAAACAGGCTCAGGAGTTAGCAAGG-3′ and 5′-TTACATTATAACGCATTTTTTAAT-3′ for *Kras*A; 5′-GAGTCTCGGCAGGCCCAGGACCTTGCTCGC-3′ and 5′-TCAGGACAGCACACATTTGCAGCT-3′ for *Hras*; 5′-GACACAAAGCAAGCCCACGAACTGGCCAAG-3′ and 5′-CTACATCAGCACACAGGGCAGCCC for *Nras*; and 5′-CTCAAGATTGTCAGCAATGC-3′ and 5′-CAAGATGCCCTTCAGTGGGC-3′ for *Gapdh*.

For assessment of the amount of each of the three Ras transcripts, amplicons formed via PCR using a primer set (5′-CAGAAICACTTTGTGGA-3′ and 5′-TTIATGGCAAATACACA-3′; I, dITP) were digested by *Dpn*II and *Pvu*II and then resolved by electrophoresis on 4% agarose gels in TBE buffer.

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

Quantitative real-time RT-PCR

Total RNA of ECs was obtained using TRIzol reagent (Invitrogen). DNase I-treated total RNA was purified and used for further study. Quantitative real-time RT-PCR was performed with the StepOne Real-Time PCR System using a High-Capacity cDNA Reverse Transcription Kit, the TaqMan Gene Expression Master Mix, the TaqMan Gene Expression Assay (*Flt4*, Mm00433337_m1), and TaqMan Endogenous control 18S rRNA (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Four full technical replicates were run and analyzed using the comparative C_T method.

Observation of chylous ascites

Newborns were checked at least once a day for 5 days after birth to determine if they had white fluid in the abdomen. Tail tips (1-2 mm) of newborns showing chylous ascites were cut for PCR genotyping and for identification of the affected mice.

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 *Vegfr3*^{EGFP} 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 $Nras^{+/-}Kras^{+/-}$ 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

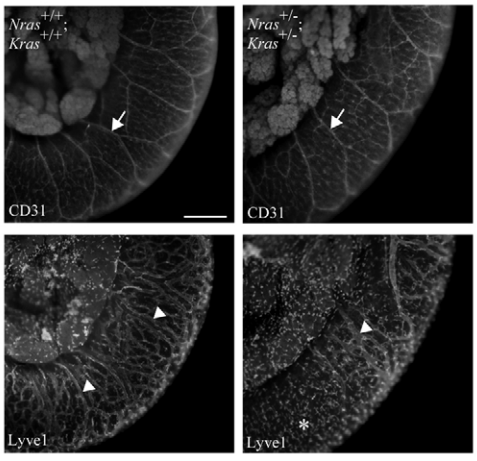
In order to determine the cell types contributing to these abnormalities, we generated transgenic mice expressing wild-type Hras in a specific cell lineage using a *Cre*/*loxP* recombination-driven expression system (see Fig. S1A in the supplementary material; hereafter referred to as CGH mice). Initially, we investigated whether endothelial expression of Hras could rescue

A

Hras	*Nras*	*Kras* +/+	*Kras* +/-
+/+	+/+	0/96 (0)	0/99 (0)
+/-		0/23 (0)	0/15 (0)
-/-		2/228 (0.9)	20/135 (14.8)
+/+	+/-	0/70 (0)	5/76 (6.6)
+/-		0/30 (0)	6/20 (30)
-/-		2/175 (1.1)	35/37 (94.6)*
+/+	-/-	2/237 (0.8)	6/6 (100)*
+/-		5/153 (3.3)	Not available
-/-		64/137 (46.7)*	Not available

B

C



D

Genotypes	*rasH2* non-Tg	*rasH2* Tg
$Hras^{-/-}; Nras^{-/-}; Kras^{+/+}$	64/137 (46.7)	0/9 (0)
$Hras^{-/-}; Nras^{+/-}; Kras^{+/-}$	35/ 37 (94.6)	0/13 (0)
$Hras^{+/+}; Nras^{-/-}; Kras^{+/-}$	6/6 (100)	0/14 (0)

E

Hras	-/-	+/+	-/-	-/-	-/-	-/-
Nras	+/-	+/+	+/+	+/-	-/-	+/-
Kras	+/-	+/+	+/+	+/+	+/+	+/-
rasH2	Tg	-	-	-	-	-

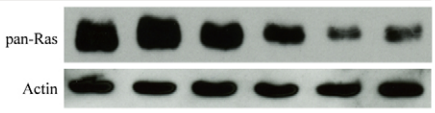


Fig. 1. Chylous ascites, caused by lymphatic vessel hypoplasia in the small intestine, of Ras compound knockout newborn mice. (**A**) Frequency of chylous ascites in Ras compound knockout newborns. Shown is the number of mice showing chylous ascites during the suckling period per total number of mice. Percentages are shown in parentheses. (**B**) Chylous ascites observed in Ras compound knockout newborns. An $Nras^{+/-}Kras^{+/-}$ mouse showing chylous ascites is shown. Asterisks indicate milk-containing stomachs. (**C**) Lymphatic vessel hypoplasia in the small intestines of newborns. Greyscale images of immunofluorescence-labelled CD31-positive blood vessels (arrows in top panels) and Lyve1-positive lymphatic vessels (arrowheads in bottom panels) obtained by double immunostaining. An asterisk indicates a region exhibiting lymphatic vessel aplasia. Scale bar: 400 μm. (**D**) The *rasH2* transgene, encoding human HRAS, rescued Ras compound knockout newborns from chylous ascites. Asterisks in A indicate the results presented in D. (**E**) Western blots showing total Ras proteins in neonatal small intestines of a variety of Ras compound knockout mice.

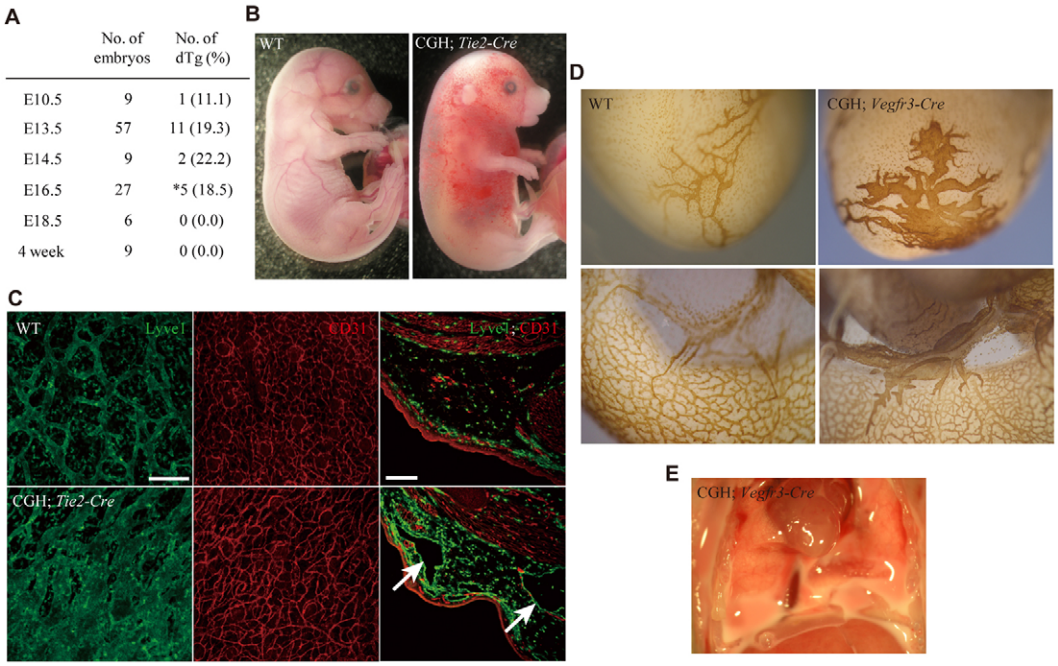


Fig. 2. Overexpression of Hras in an endothelial cell lineage leads to lymphatic vessel hyperplasia in mice. (**A**) CGH mice were crossed with *Tie2-Cre* mice and the resulting embryos were examined at several embryonic stages. CGH/*Tie2-Cre* double-transgenic (dTg) embryos died by E16.5. The asterisk indicates the number of dead embryos in utero. (**B**) CGH/*Tie2-Cre* double-transgenic embryos showed cutaneous edema at E15.5. WT, wild-type. (**C**) Lymphatic vessels were dilated and hyperplastic in the skin of CGH/*Tie2-Cre* double-transgenic embryos at E15.5. The wholemount skin (left and middle panels) and sectioned skin (right panels) were double-labelled for Lyve1 (green) and CD31 (red). Arrows indicate dilated lymphatic vessels. (**D**) Lymphatic vessel hyperplasia in the heart (upper panels), the mesentery and intestinal serosa (lower panels) of CGH/*Vegfr3-Cre* double-transgenic adult mice expressing the *Hras*-IRES-EGFP transgene. Lyve1-positive lymphatic vessels are labelled in brown. (**E**) Chylothorax in CGH/*Vegfr3-Cre* double-transgenic mice. Scale bars: 300 μm for wholemounts; 200 μm for sections.

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. S2B 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). Lyve1 and mutated SV40T antigen-positive mesenteric LECs were obtained from 2-week-old, T26/*Tie2-Cre* double- and T26/CGH/*Vegfr3-Cre* triple-transgenic mice (see Fig. S3A,B in the supplementary material). Western blot analysis showed that 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/CGH/*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 *Kras*B transcripts were higher than those of *Kras*A, 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

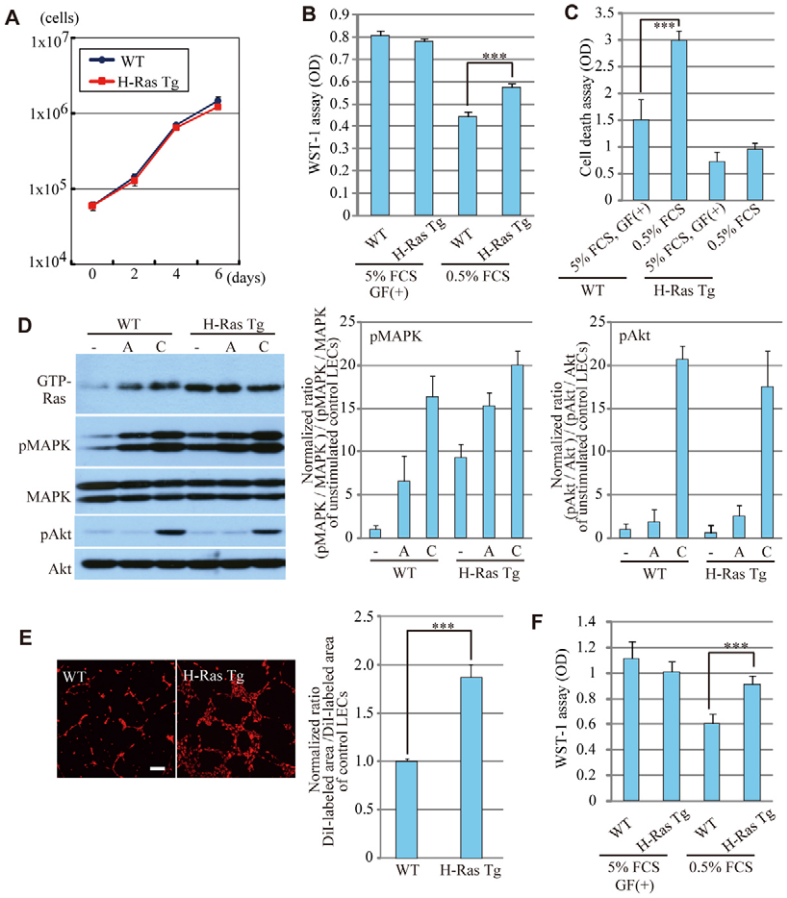


Fig. 3. Characterization of Hras-overexpressing LECs in vitro. (**A**) Proliferation of LECs in normal cell culture conditions for 6 days. Error bars, s.d., *n*=3. (**B**) WST-1 assay. LECs were cultured on gelatinized dishes in normal or serum- and growth factor-depleted media. Error bars, s.d., *n*=12. ***, *P*<0.005. (**C**) Cell death assay. 2×10^4 cells were cultured in normal (*n*=3 each) or serum- and growth factor-depleted media (*n*=4 each) for 24 hours. ***, *P*<0.005. (**D**) Western blots and quantitative analysis of activation of MAPKs and Akt in LECs. –, no stimulation; A, VEGFA stimulation; C, VEGFC stimulation; H-Ras, Hras-overexpressing LECs; WT, wild-type LECs; GTP-Ras, GTP-bound Ras precipitated using Raf-RBD. Anti-pMAPK and anti-pAkt reveal the levels of phosphorylated MAPK and Akt, respectively. MAPK and Akt show total protein levels. Error bars, s.d., *n*=4. (**E**) DiI-stained cellular networks of LECs on Matrigel. Sheet formation of LECs was induced by Ras overexpression. DiI-labelled areas were quantified and the mean area of DiI-labelled wild-type LECs was normalized to 1. Error bars, s.d., *n*=3. ***, *P*<0.005. Scale bar: 200 μm. (**F**) WST-1 assay. LECs were cultured for 24 hours on Matrigel in normal or serum- and growth factor-depleted media. Error bars, s.d., *n*=8. ***, *P*<0.005.

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*, *Kras*B and then *Kras*A (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.

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. S5D 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* open reading frame sequence of the transgene in mice or LECs (data not shown), and Ras does not intrinsically bind GTP, it is probable that the elevated activation of overexpressed Hras in starved LECs occurred through external growth factors in the foetal calf serum or perhaps autocrine growth factors.

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

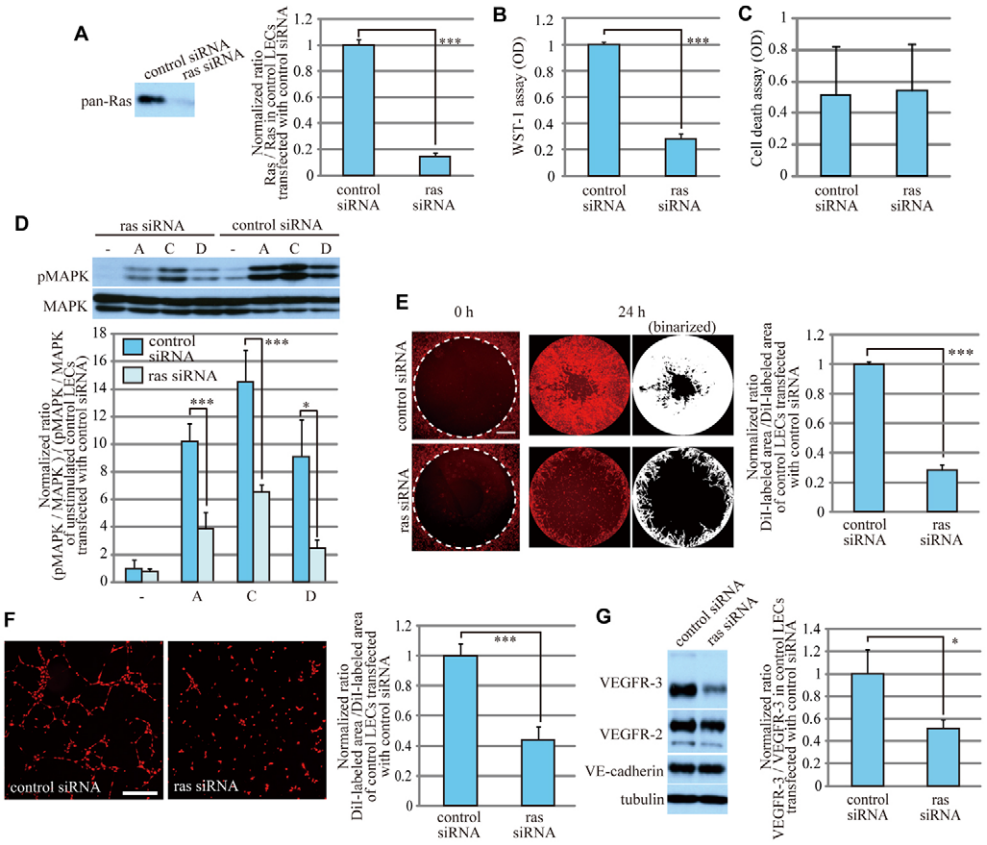


Fig. 4. Characterization of Ras knockdown LECs in vitro. (**A**) Transfection of mixed siRNAs for *Hras*, *Nras*, and *Kras* genes into wild-type LECs downregulates the expression of Ras proteins. Left, A western blot; right, quantitative analysis of Ras protein expression. Error bars, s.d., *n*=3. ***, *P*<0.005. (**B**) WST-1 assay. Error bars, s.d., *n*=12. ***, *P*<0.005. (**C**) Cell death assay. Error bars, s.d., *n*=8. LECs were cultured on gelatinized dishes in a normal culture condition for B and C. (**D**) Ras-mediated MAPK activation by VEGFs was impaired by the knockdown of Ras proteins. –, no stimulation; A, VEGFA stimulation; C, VEGFC stimulation; D, VEGFD stimulation. Upper, western blots; lower, quantitative analysis of activation of MAPKs. Error bars, s.d., *n*=3. *, *P*<0.05, ***, *P*<0.005. (**E**) Cell migration assay. Dil-labelled areas were quantified. The mean area of wild-type LECs transfected with control siRNA was normalized to 1. Error bars, s.d., *n*=10. ***, *P*<0.005. Scale bar: 400 μm. (**F**) Network formation of LECs was blocked by the knockdown of Ras proteins. Dil-labelled areas were quantified and the mean area of wild-type LECs transfected with control siRNA was normalized to 1. Error bars, s.d., *n*=3. ***, *P*<0.005. Scale bar: 400 μm. (**G**) Western blots and quantitative analysis of VEGFR3 protein expression. Ras knockdown reduces VEGFR3 protein expression in LECs. The endothelial markers VEGFR2 and VE-cadherin were shown for comparison. Tubulin is used as a loading control. Error bars, s.d., *n*=3. *, *P*<0.05.

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 VEGFD, stimulating only mouse VEGFR3 in contrast to VEGFC and human VEGFD, 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 VEGFR3 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

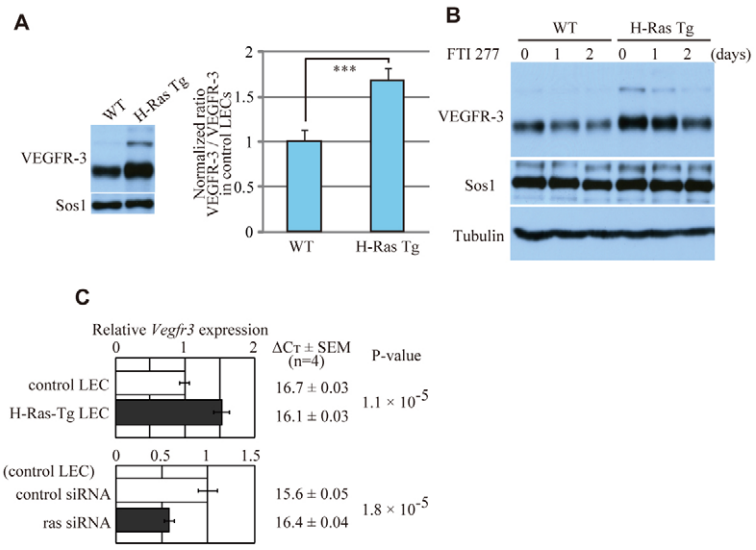


Fig. 5. VEGFR3 expression correlates with Ras expression level and its activation. (**A**) VEGFR3 expression is higher in Hras-overexpressing LECs than control LECs. Sos1 was chosen as an indicator of transfer of high molecular weight proteins. Error bars, s.d., n=3. ***, P<0.005. (**B**) FTI treatment downregulates VEGFR3 expression in LECs. Sos1 and Tubulin are used as loading controls. (**C**) Real-time RT-PCR assay for *Vegfr3* expression. Ras overexpression and knockdown lead to transcriptional up- and downregulation of the *Vegfr3* gene, respectively.

(Kirkin et al., 2001). 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. Kinase-deficient heterozygous mutations of *Vegfr3* cause lymphatic vessel hypoplasia and primary lymphedema in humans and 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). Ras compound knockout mice in the B6 genetic background are susceptible to chylous ascites (73%, 19 out of 26 $Nras^{+/-}Kras^{+/-}$ mice; data not shown for others), whereas mice heterozygous-null for either *Nras* or *Kras* never developed chylous ascites (0 out of 40 $Nras^{+/-}$ and 27 $Kras^{+/-}$ mice; Fig. 7A). When crossing $Kras^{+/-}$ mice with $Vegfr3^{+/-}$ mice, almost all of the resulting $Kras^{+/-}Vegfr3^{+/-}$ newborns exhibited 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).

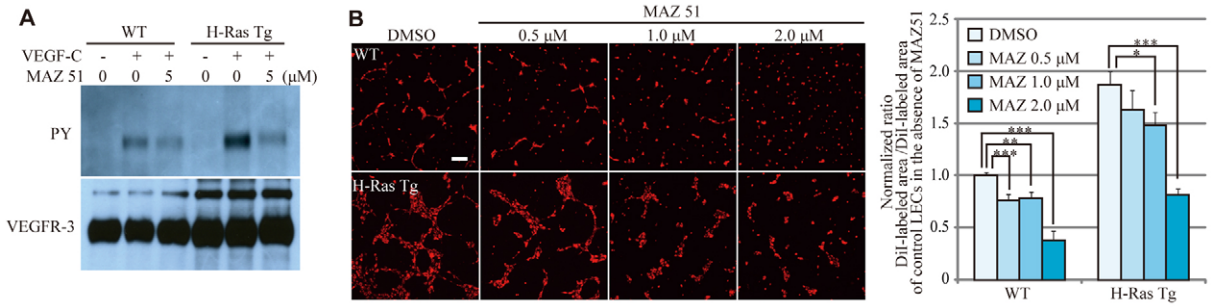


Fig. 6. Endothelial sheet formation of Hras-overexpressing LECs is dependent on VEGFR3 signalling. (**A**) Western blots of LEC proteins immunoprecipitated by anti-VEGFR3. MAZ51, a selective kinase inhibitor of VEGFR3, blocked VEGFC-induced phosphorylation of VEGFR3. PY, phosphorylated tyrosine. (**B**) Network and sheet formation of LECs was blocked by MAZ51 in a dose-dependent manner. DiI-labelled cells (red) were imaged and the areas were analyzed. A mean area of wild-type LECs in the presence of DMSO was designated as 1. Error bars, s.d., n=3. *, P<0.05; **, P<0.01; ***, P<0.005. Scale bar: 200 μm.

A

Genotype	No. newborns showing chylous ascites / No. newborns observed
Vegfr3$^{+/EGFP}$; *Kras*$^{+/+}$	7/28 (25%)
Vegfr3$^{+/+}$; *Kras*$^{+/-}$	0/27 (0%)
Vegfr3$^{+/EGFP}$; *Kras*$^{+/-}$	16/16 (100%)
Vegfr3$^{+/EGFP}$; *Kras*$^{+/+}$; *rasH2* Tg	0/21 (0%)
Vegfr3$^{+/EGFP}$; *Kras*$^{+/-}$; *rasH2* Tg	1/13 (7.7%)

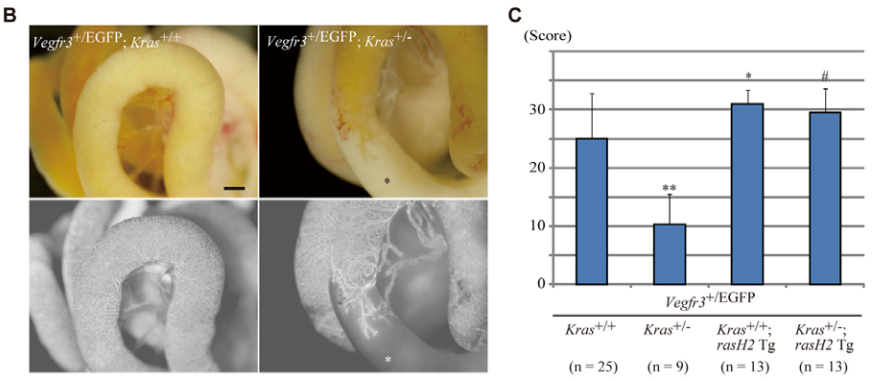


Fig. 7. The Ras genes modify VEGFR3-dependent lymphatic vessel growth in a gene dose-dependent manner. (**A**) Frequency of chylous ascites observed in newborns carrying a *Vegfr3*^{EGFP} knock-in allele in the presence or absence of a *Kras*-null allele and/or a *rasH2* transgenic allele. (**B**) EGFP-expressing lymphatic vessels (labelled in greyscale images of lower panels) in the small intestine of newborns carrying a *Vegfr3*^{EGFP} 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 *Vegfr3*$^{+/EGFP}$*Kras*$^{+/+}$ mice); *, $P<0.05$ (versus *Vegfr3*$^{+/EGFP}$*Kras*$^{+/+}$ mice); #, $P<0.000001$ (versus *Vegfr3*$^{+/EGFP}$*Kras*$^{+/-}$ mice).

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

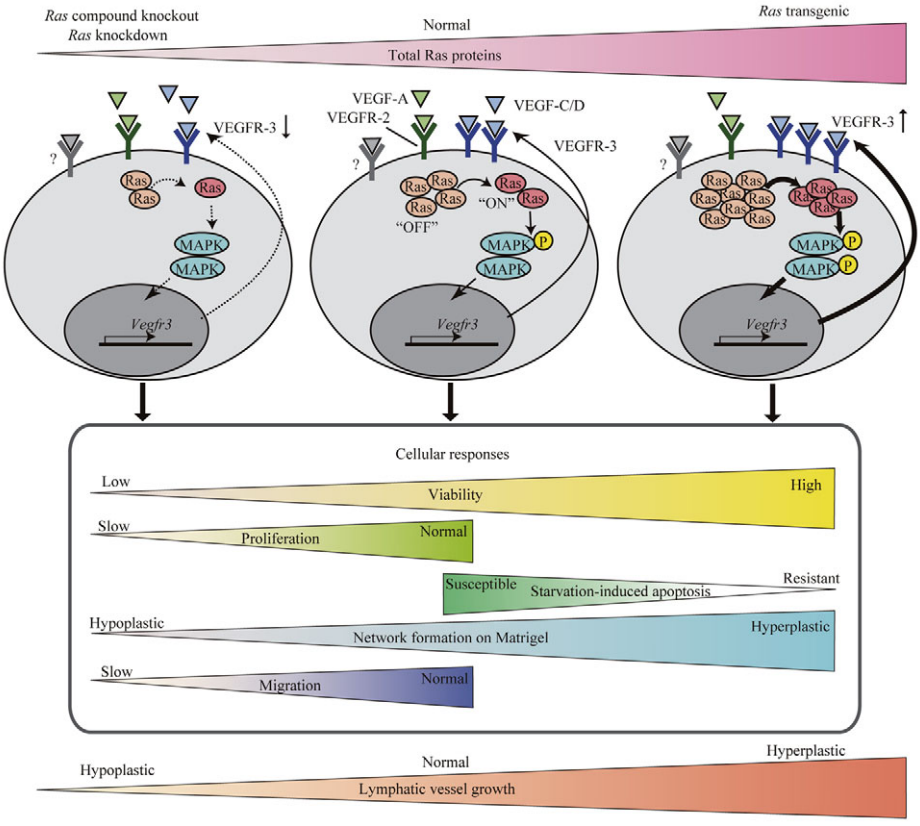


Fig. 8. Schematic representation of the role of Ras in lymphangiogenesis. Ras protein expression levels in LECs not only influence GTP-bound Ras levels and MAPK activity levels as regulated by upstream signals via VEGFR2, VEGFR3 and other kinases, but also VEGFR3 expression levels. The increase or decrease 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 VEGF3 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 *Spred1* and *Spred2* 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 VEGFC/VEGFR3 signalling by Spred 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 *Pi3kca* 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*^{Chy}) mice showing chylous ascites and lymphedema (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 lymphedema. 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, lymphedema, lymphangioma/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.

Supplementary material
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References
Adams, R. H. and Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. *Nat. Rev. Mol. Cell Biol.* **8**, 464-478.
Aoki, Y., Niihori, T., Kawame, H., Kurosawa, K., Ohashi, H., Tanaka, Y., Filocamo, M., Kato, K., Suzuki, Y., Kure, S. et al. (2005). Germline mutations in HRAS proto-oncogene cause Costello syndrome. *Nat. Genet.* **37**, 1038-1040.
Ayadi, A., Zheng, H., Sobieszczuk, P., Buchwalter, G., Moerman, P., Alitalo, K. and Wasylyk, B. (2001). Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. *EMBO J.* **20**, 5139-5152.
Baldwin, M. E., Catimel, B., Nice, E. C., Roufail, S., Hall, N. E., Stenvers, K. L., Karkkainen, M. J., Alitalo, K., Stacker, S. A. and Achen, M. G. (2001). The specificity of receptor binding by vascular endothelial growth factor-d is different in mouse and man. *J. Biol. Chem.* **276**, 19166-19171.
Chan, P. C., Chiu, H. C. and Hwu, W. L. (2002). Spontaneous chylothorax in a case of cardio-facio-cutaneous syndrome. *Clin. Dysmorphol.* **11**, 297-298.
Cirstea, I. C., Kutsche, K., Dvorsky, R., Gremer, L., Carta, C., Horn, D., Roberts, A. E., Lepri, F., Merbitz-Zahradnik, T., Konig, R. et al. (2010). A restricted spectrum of NRAS mutations causes Noonan syndrome. *Nat. Genet.* **42**, 27-29.
Donnenfeld, A. E., Nazir, M. A., Sindoni, F. and Librizzi, R. J. (1991). Prenatal sonographic documentation of cystic hygroma regression in Noonan syndrome. *Am. J. Med. Genet.* **39**, 461-465.
Esteban, L. M., Vicario-Abejon, C., Fernandez-Salguero, P., Fernandez-Medarde, A., Swaminathan, N., Yienger, K., Lopez, E., Malumbres, M., McKay, R., Ward, J. M. et al. (2001). Targeted genomic disruption of H-ras and N-ras, individually or in combination, reveals the dispensability of both loci for mouse growth and development. *Mol. Cell. Biol.* **21**, 1444-1452.
Evans, D. G., Lonsdale, R. N. and Patton, M. A. (1991). Cutaneous lymphangioma and amegakaryocytic thrombocytopenia in Noonan syndrome. *Clin. Genet.* **39**, 228-232.
Gupta, S., Ramjaun, A. R., Haiko, P., Wang, Y., Warne, P. H., Nicke, B., Nye, E., Stamp, G., Alitalo, K. and Downward, J. (2007). Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. *Cell* **129**, 957-968.
Haiko, P., Makinen, T., Keskitalo, S., Taipale, J., Karkkainen, M. J., Baldwin, M. E., Stacker, S. A., Achen, M. G. and Alitalo, K. (2008). Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. *Mol. Cell. Biol.* **28**, 4843-4850.
Henkemeyer, M., Rossi, D. J., Holmyard, D. P., Puri, M. C., Mbamalu, G., Harpal, K., Shih, T. S., Jacks, T. and Pawson, T. (1995). Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein. *Nature* **377**, 695-701.
Ichise, H., Ichise, T., Ohtani, O. and Yoshida, N. (2009). Phospholipase Cgamma2 is necessary for separation of blood and lymphatic vasculature in mice. *Development* **136**, 191-195.
Ise, K., Nakamura, K., Nakao, K., Shimizu, S., Harada, H., Ichise, T., Miyoshi, J., Gondo, Y., Ishikawa, T., Aiba, A. et al. (2000). Targeted deletion of the H-ras gene decreases tumor formation in mouse skin carcinogenesis. *Oncogene* **19**, 2951-2956.

Johnson, L., Greenbaum, D., Cichowski, K., Mercer, K., Murphy, E., Schmitt, E., Bronson, R. T., Umanoff, H., Edelmann, W., Kucherlapati, R. et al. (1997). K-ras is an essential gene in the mouse with partial functional overlap with N-ras. *Genes Dev.* **11**, 2468-2481.
Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N. and Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* **15**, 290-298.
Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V. W., Fang, G. H., Dumont, D., Breitman, M. and Alitalo, K. (1995). Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci. USA* **92**, 3566-3570.
Karkkainen, M. J., Ferrell, R. E., Lawrence, E. C., Kimak, M. A., Levinson, K. L., McTigue, M. A., Alitalo, K. and Finegold, D. N. (2000). Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat. Genet.* **25**, 153-159.
Karkkainen, M. J., Saaristo, A., Jussila, L., Karila, K. A., Lawrence, E. C., Pajusola, K., Bueler, H., Eichmann, A., Kauppinen, R., Kettunen, M. I. et al. (2001). A model for gene therapy of human hereditary lymphedema. *Proc. Natl. Acad. Sci. USA* **98**, 12677-12682.
Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H. et al. (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* **5**, 74-80.
Karnoub, A. E. and Weinberg, R. A. (2008). Ras oncogenes: split personalities. *Nat. Rev. Mol. Cell Biol.* **9**, 517-531.
Karpanen, T. and Alitalo, K. (2008). Molecular biology and pathology of lymphangiogenesis. *Annu. Rev. Pathol.* **3**, 367-397.
Kawasaki, K., Watabe, T., Sase, H., Hirashima, M., Koide, H., Morishita, Y., Yuki, K., Sasaoka, T., Suda, T., Katsuki, M. et al. (2008). Ras signaling directs endothelial specification of VEGFR2+ vascular progenitor cells. *J. Cell Biol.* **181**, 131-141.
Khalaf, W. F., White, H., Wenning, M. J., Orazi, A., Kapur, R. and Ingram, D. A. (2005). K-Ras is essential for normal fetal liver erythropoiesis. *Blood* **105**, 3538-3541.
Kirkin, V., Mazitschek, R., Krishnan, J., Steffen, A., Waltenberger, J., Pepper, M. S., Giannis, A. and Sleeman, J. P. (2001). Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. *Eur. J. Biochem.* **268**, 5530-5540.
Koera, K., Nakamura, K., Nakao, K., Miyoshi, J., Toyoshima, K., Hatta, T., Otani, H., Aiba, A. and Katsuki, M. (1997). K-ras is essential for the development of the mouse embryo. *Oncogene* **15**, 1151-1159.
Koni, P. A., Joshi, S. K., Temann, U. A., Olson, D., Burkly, L. and Flavell, R. A. (2001). Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *J. Exp. Med.* **193**, 741-754.
Mao, X., Fujiwara, Y. and Orkin, S. H. (1999). Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc. Natl. Acad. Sci. USA* **96**, 5037-5042.
Matsui, T., Kinoshita, T., Morikawa, Y., Tohya, K., Katsuki, M., Ito, Y., Kamiya, A. and Miyajima, A. (2002). K-Ras mediates cytokine-induced formation of E-cadherin-based adherens junctions during liver development. *EMBO J.* **21**, 1021-1030.
Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593-605.
Nakamura, K., Ichise, H., Nakao, K., Hatta, T., Otani, H., Sakagami, H., Kondo, H. and Katsuki, M. (2008). Partial functional overlap of the three ras genes in mouse embryonic development. *Oncogene* **27**, 2961-2968.
Niihori, T., Aoki, Y., Narumi, Y., Neri, G., Cave, H., Verloes, A., Okamoto, N., Hennekam, R. C., Gillessen-Kaesbach, G., Wieczorek, D. et al. (2006). Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. *Nat. Genet.* **38**, 294-296.
Niwa, H., Yamamura, K. and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199.
Pells, S., Divjak, M., Romanowski, P., Impey, H., Hawkins, N. J., Clarke, A. R., Hooper, M. L. and Williamson, D. J. (1997). Developmentally regulated expression of murine K-ras isoforms. *Oncogene* **15**, 1781-1786.
Plowman, S. J., Williamson, D. J., O'Sullivan, M. J., Doig, J., Ritchie, A. M., Harrison, D. J., Melton, D. W., Arends, M. J., Hooper, M. L. and Patek, C. E. (2003). While K-ras is essential for mouse development, expression of the K-ras 4A splice variant is dispensable. *Mol. Cell. Biol.* **23**, 9245-9250.
Potenza, N., Vecchione, C., Notte, A., De Rienzo, A., Rosica, A., Bauer, L., Affuso, A., De Felice, M., Russo, T., Poulet, R. et al. (2005). Replacement of K-Ras with H-Ras supports normal embryonic development despite inducing cardiovascular pathology in adult mice. *EMBO Rep.* **6**, 432-437.
Prevo, R., Banerji, S., Ferguson, D. J., Clasper, S. and Jackson, D. G. (2001). Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J. Biol. Chem.* **276**, 19420-19430.
Saitoh, A., Kimura, M., Takahashi, R., Yokoyama, M., Nomura, T., Izawa, M., Sekiya, T., Nishimura, S. and Katsuki, M. (1990). Most tumors in transgenic mice with human c-Ha-ras gene contained somatically activated transgenes. *Oncogene* **5**, 1195-1200.
Schubbert, S., Zenker, M., Rowe, S. L., Boll, S., Klein, C., Bollag, G., van der Burgt, I., Musante, L., Kalscheuer, V., Wehner, L. E. et al. (2006). Germline KRAS mutations cause Noonan syndrome. *Nat. Genet.* **38**, 331-336.
Serban, D., Leng, J. and Cheresh, D. (2008). H-ras regulates angiogenesis and vascular permeability by activation of distinct downstream effectors. *Circ. Res.* **102**, 1350-1358.
Takeuchi, T., Nomura, T., Tsujita, M., Suzuki, M., Fuse, T., Mori, H. and Mishina, M. (2002). Flp recombinase transgenic mice of C57BL/6 strain for conditional gene targeting. *Biochem. Biophys. Res. Commun.* **293**, 953-957.
Taniguchi, K., Kohno, R., Ayada, T., Kato, R., Ichiyama, K., Morisada, T., Oike, Y., Yonemitsu, Y., Maehara, Y. and Yoshimura, A. (2007). Spreds are essential for embryonic lymphangiogenesis by regulating vascular endothelial growth factor receptor 3 signaling. *Mol. Cell. Biol.* **27**, 4541-4550.
Wasylyk, B., Hagman, J. and Gutierrez-Hartmann, A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem. Sci.* **23**, 213-216.
Whyte, D. B., Kirschmeier, P., Hockenberry, T. N., Nunez-Oliva, I., James, L., Catino, J. J., Bishop, W. R. and Pai, J. K. (1997). K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J. Biol. Chem.* **272**, 14459-14464.
Wigle, J. T. and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. *Cell* **98**, 769-778.
Witt, D. R., Hoyme, H. E., Zonana, J., Manchester, D. K., Fryns, J. P., Stevenson, J. G., Curry, C. J. and Hall, J. G. (1987). Lymphedema in Noonan syndrome: clues to pathogenesis and prenatal diagnosis and review of the literature. *Am. J. Med. Genet.* **27**, 841-856.
Wojnowski, L., Zimmer, A. M., Beck, T. W., Hahn, H., Bernal, R., Rapp, U. R. and Zimmer, A. (1997). Endothelial apoptosis in Braf-deficient mice. *Nat. Genet.* **16**, 293-297.
Wu, M., Wallace, M. R. and Muir, D. (2006). Nf1 haploinsufficiency augments angiogenesis. *Oncogene* **25**, 2297-2303.
Xu, K., Chong, D. C., Rankin, S. A., Zorn, A. M. and Cleaver, O. (2009). Rasip1 is required for endothelial cell motility, angiogenesis and vessel formation. *Dev. Biol.* **329**, 269-279.
Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y. and Aizawa, S. (1993). A novel negative selection for homologous recombinants using diphtheria toxin A fragment gene. *Anal. Biochem.* **214**, 77-86.
Yamaguchi, T., Ichise, T., Iwata, O., Hori, A., Adachi, T., Nakamura, M., Yoshida, N. and Ichise, H. (2008). Development of a new method for isolation and long-term culture of organ-specific blood vascular and lymphatic endothelial cells of the mouse. *FEBS J.* **275**, 1988-1998.