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Heterozygous expression of the oncogenic *Pik3ca*^{H1047R} mutation during murine development results in fatal embryonic and extraembryonic defects

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ABSTRACT

The phosphoinositide 3-kinase (PI3K)/AKT signalling pathway regulates many cellular functions including proliferation, migration, survival and protein synthesis. Somatic mutations in *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K enzyme, are commonly associated with many human cancers as well as recently being implicated in human overgrowth syndromes. However, it is not clear if such mutations can be inherited through the germline. We have used a novel mouse model with Cre recombinase (Cre)-conditional knock-in of the common H1047R mutation into the endogenous *Pik3ca* gene. Heterozygous expression of the *Pik3ca*^{H1047R} mutation in the developing mouse embryo resulted in failed 'turning' of the embryo and disrupted vascular remodelling within the embryonic and extraembryonic tissues, leading to lethality prior to E10. As vascular endothelial growth factor A (VEGF-A) signalling was disrupted in these embryos, we used Cre under the control of the Tie2 promoter to target the *Pik3ca*^{H1047R} mutation specifically to endothelial cells. In these embryos turning occurred normally but the vascular remodelling defects and embryonic lethality remained, likely as a result of endothelial hyperproliferation. Our results confirm the lethality associated with heterozygous expression of the *Pik3ca*^{H1047R} mutation during development and likely explain the lack of inherited germline *PIK3CA* mutations in humans.

Introduction

The phosphoinositide 3-kinase (PI3K)/AKT signalling pathway regulates processes critical for cell proliferation, growth, migration, survival and angiogenesis (Vanhaesebroeck et al., 2012). The p110α catalytic subunit of class 1A PI3Ks is encoded by the gene *PIK3CA*. Somatic mutations within this gene are commonly observed in many human cancers including breast and colon cancers (Samuels and Waldman, 2010; Zardavas et al., 2014). The majority of *PIK3CA* mutations cluster at three hot spots within the protein, two in the helical domain (E542 and E545) and one in the kinase domain (H1047), and cause the constitutive activation of the PI3K/Akt signalling pathway (reviewed in Chalhoub and Baker (2009)). Interestingly, similar mutations, including the common cancer-associated 'hot spot' mutations, have also recently been observed in overgrowth conditions such as megalencephaly syndromes (Lee et al., 2012; Riviere et al., 2012), fibroadipose hyperplasia (Lindhurst et al., 2012) and Congenital Lipomatous Overgrowth with Vascular, Epidermal, and Skeletal anomalies (CLOVES) (Kurek et al., 2012).

Cancer-associated *PIK3CA* mutations are invariably somatic and PIK3CA-driven overgrowth syndromes are not inherited suggesting that *PIK3CA* mutations are not compatible with germline transmission. Consistent with this, germline homozygous loss of phosphatase and tensin homologue (*Pten*), a negative regulator of PI3K, during murine development is fatal before embryonic day (E) 11.5 due to vascular abnormalities resulting in bleeding and cardiac failure that arise from aberrant endothelial cell (EC) proliferation (Hamada et al., 2005).

Interestingly, mice lacking the p110α subunit (Bi et al., 1999; Lelievre et al., 2005), or expressing a kinase-dead version of the

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p110α isoform (Graupera et al., 2008), are also embryonic lethal, albeit at slightly varying stages of mid-gestation. Notably, in both cases embryonic lethality is suggested to arise from impaired EC development in which cell migration, proliferation and survival are variably affected. Mice expressing the kinase-dead p110α specifically in ECs demonstrated similar migration and motility defects with lethality occurring before E12.5 (Graupera et al., 2008). Similarly, the combined ablation of both *Akt1* and *Akt3* results in embryonic lethality at approximately E11 caused by a decreased vasculature and other cardiovascular defects as well as increased apoptosis in the developing nervous system (Yang et al., 2005). However, $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ embryos are able to survive embryogenesis (Dummler et al., 2006) suggesting that *Akt1*, in particular, is essential for embryogenic development.

These mouse models indicate that tight regulation of PI3K/AKT pathway activity is essential during embryonic development suggesting that mutations which disrupt the normal physiological activity of PIK3CA would be unlikely to be heritable. Nevertheless, germline transmission of *PIK3CA* mutations has recently been reported in 8 individuals with Cowden or "Cowden-like" syndrome (Orloff Mohammed et al., 2013). However, the reported mutations are not within known oncogenic 'hot spot' regions in the *PIK3CA* gene.

In order to determine if the cancer-causing *PIK3CA* 'hot spot' mutations can indeed be transmitted through the germline, we have used a mouse model with a Cre recombinase (Cre)-conditional knock-in of the common cancer-associated H1047R mutation into the endogenous *Pik3ca* gene (Kinross et al., 2012; Tikoo et al., 2012; Hare et al., 2014). We show that both ubiquitous and EC-specific expression of the $Pik3ca^{H1047R}$ mutation during embryonic development lead to severe vascular malformations that result in lethality prior to E10.5.

Materials and methods

Generation of mice

$Pik3ca^{Lat\text{-}H1047R}$ mice, generated as previously described (Kinross et al., 2012), were mated to either *Cre-deleter* (B6.C-Tg(CMV-cre)1Cgn/J) transgenic mice which ubiquitously express Cre recombinase (Cre) from the zygote stage of development (Schwenk et al., 1995) or *Tie2Cre* (B6.Cg-Tg(Tek-cre)1Ywa/J) transgenic mice whose Cre expression is under the control of the EC-specific *Tie2* promoter (Kisanuki et al., 2001). All mice strains were maintained on the C57BL/6 background. For crosses involving the *Cre-deleter* strain, female $Cre\text{-}deleter^{+/-}$ mice were crossed with male $Pik3ca^{Lat\text{-}H1047R/WT}$ mice. The $Rosa^{LacZ}$ (B6;129S4-*Gt*(ROSA)*26Sor*^{tm1Sor}/J) transgenic reporter mouse strain (Soriano, 1999) was used for verification of Cre-mediated recombination in both the *Cre-deleter* and *Tie2Cre* embryos.

Offspring heterozygous for either *Cre-deleter* (*Cre-del), Tie2Cre (Tie2Cre)* or $Pik3ca^{H1047R}$ alone (*Pik3ca*) were classified as controls while those heterozygous for both *Cre-deleter* and $Pik3ca^{H1047R}$ (*Cre-del*:*Pik3ca*) or *Tie2Cre* and $Pik3ca^{H1047R}$ (*Tie2Cre:Pik3ca*) were classified as mutants.

All experimental procedures involving animals were approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee and conducted in accordance with the National Health and Medical Research Council (NHMRC) *Australian Code of Practise for the Care and Use of Animals for Scientific Purposes*.

Embryo collection

Blastocysts were collected at E3.5 as previously described (Nagy et al., 2003a). Briefly, *Cre-del* female mice were injected with 5 i.u. of pregnant mare's serum gonadotropin (PMS: Folligon, Intervet) followed by 2.5 i.u. of human chorionic gonadotropin (hCG: Chorulon, Intervet) 48 h later, to stimulate ovulation. This was followed by overnight mating with a male $Pik3ca^{Lat\text{-}H1047R/WT}$ mouse. Two days later, pregnant females were culled and E2.5 morula collected and placed in culture overnight at 6% CO_2 in Cooks blastocyst media (Cook Medical, Australia) before DNA was extracted from individual E3.5 embryos for genotyping.

Embryos were obtained from timed matings with the day of the detection of a vaginal plug considered as E0.5. Embryos and yolk sacs between E8.5 and E14.5 were isolated as previously described (Nagy et al., 2003b). Yolk sacs were taken for DNA isolation and genotyping when possible. Alternatively, when yolk sacs were required, embryo tails were used for genotyping.

PCR genotyping of embryos

Embryo tails or yolk sacs were placed in cell lysis buffer consisting of 50 mM Tris pH 8.8, 10 mM $(NH_4)SO_4$, 6.5 mM $MgCl_2$, 0.5% Triton X-100 and 1% β-mercaptoethanol (200 μL for E10–14.5, 100 μL for E8–9.5 and 20 μL for E3.5 embryos) and proteinase-K added to a final concentration of 150 μg/mL. Following overnight incubation at 55 °C, samples were heated to 95 °C for 15 min, mixed well and centrifuged at 13,000 rpm for 5 min. The primers for analysis of *Cre* and *Pik3ca* genotypes, and PCR conditions for each reaction, were as previously described (Kinross et al., 2012).

Immunofluorescence and LacZ staining

Freshly isolated embryos and yolk sacs were fixed in cold 4% paraformaldehyde at 4 °C for 2 h. For immunolabelling, cryosections or wholemount embryos were blocked in 10% (v/v) goat serum, 0.2% (w/v) BSA, 0.2% (v/v) Triton X-100 in phosphate-buffered saline (PBS), and stained with the indicated primary antibodies overnight at 4 °C. Antibodies used included: rabbit anti-cleaved caspase 3 (Cell Signalling Technology, Danvers, MA) 1:500; rabbit anti-phospho-Histone H3 (Cell Signalling Technology) 1:500; rat anti-endomucin (Santa Cruz, Dallas, TX) 1:50; goat anti-Nrp1 (R&D, Minneapolis, MN) 1:500; rabbit anti-Nrp2 (Cell Signalling Technology) 1:500; rat anti-PECAM/CD31 (Biolegend, San Diego, CA) 1:200; goat anti-VEGFR (R&D, Minneapolis, MN) 1:200; mouse anti-smooth muscle actin (Sigma) 1:500. Cyrosections and cells were mounted in Prolong Gold antifade reagent with DAPI (Molecular Probes, Mulgrave, Australia). Confocal images were acquired on a LSM 700 (Zeiss) system.

For *LacZ* staining, embryos were fixed in cold PBS containing 2% (v/v) formalin and 0.2% (v/v) gluteraldehyde for 2 h at 4 °C. Embryos were then rinsed in PBS before being immersed in staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and 1 mg/mL X-Gal in PBS) for 6–12 h at 37 °C.

RNA isolation and RT-PCR

RNA was extracted from E9.5 embryos using an RNeasy Mini kit (Qiagen) and reverse transcribed using the Transcriptor First Strand cDNA Synthesis kit (Roche). The QuantiTect Primer Assays (Qiagen) were used in combination with the QuantiTect SYBR Green RT-PCR kit (Qiagen) for analysis of the expression of various vascular growth factors (VGFs). Qiagen Quantitect primers used included *EphrinB2* (cat. #QT00139202), *Tgfβ* (cat. #QT00135828), *Vegf-A* (cat. #QT00160769), *Vegf-C* (cat. #QT00104027), *Vegfr1* (*Flt1* – cat. #QT00096292), *Vegfr2* (*Flk1* – cat. #QT00097020), *Vegfr3* (*Flt4* – cat. #QT00099064), *Ang1* (cat. #QT00143381), *Ang2* (cat. #QT00326256), *Nrp1* (cat. #QT00157381) and *Nrp2* (cat. #QT00154427). Primers were designed for *Foxo1* (sense 5′-TGTCAGGCTAAGAGTTAGTGAGCA-3′ and antisense 5′-GGGTGAAGGGGCATCTTTG-3′) and *Foxo3a* (sense 5′-AGCAGGCCTCATCAAAG-3′ and