Oncogenic G Protein GNAQ Induces Uveal Melanoma and Intravasation in Mice

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Abstract

GNAQ and GNA11 are heterotrimeric G protein alpha subunits, which are mutated in a mutually exclusive pattern in most cases of uveal melanoma, one of the most aggressive cancers. Here we introduce the first transgenic mouse model of uveal melanoma, which develops cancers induced by expression of oncogenic GNAQ\(^{Q209L}\) under control of the Rosa26 promoter. Disease penetrance is 100% by 3 months of age, with 94% of mice also developing lung tumors. In this model, the Yap protein of the Hippo pathway is activated in the eyes, and blood vessels near the lesions in the head and lungs exhibit melanocytic invasion. While full transcription levels are not achieved, identifying a novel role for GNAQ in melanocyte-like cells, we obtained suggestive evidence of a selective advantage for increased GNAQ\(^{Q209L}\) expression in human tumors. Intriguingly, enforced expression of GNAQ\(^{Q209L}\) progressively eliminated melanocytes from the interfollicular epidermis in adults, possibly explaining the near absence of GNAQ\(^{Q209L}\) mutations in human epithelial melanomas. The mouse model also exhibited dermal nevi and melanocytic neoplasms of the central nervous system, accompanied by impaired hearing and balance, identifying a novel role for GNAQ in melanocyte-like cells of the inner ear. Overall, this model offers a new tool to dissect signaling by oncogenic GNAQ and to test potential therapeutics in an in vivo setting where GNAQ\(^{Q209L}\) mutations contribute to both the initiation and metastatic progression of uveal melanoma. Cancer Res; 75(16); 1–14. ©2015 AACR.

Introduction

Uveal melanoma, a malignancy of melanocytes in the uveal tract of the eye, is a highly aggressive cancer without any effective treatment options once it metastasizes (1). Large-scale uveal melanoma sequencing projects have identified recurrent mutations in two G proteins (GNAQ and GNA11), BRC1-associated protein-1 (BAP1), X-linked eukaryotic translation initiation factor 1A (EIF1AX), and Splicing factor 3B subunit 1 (SF3B1; refs. 2–8). Of these, mutations in GNAQ and GNA11 occur with a mutually exclusive, combined frequency of approximately 80% in both class I and the more aggressive class II uveal melanomas (2, 7). There are two oncogenic hotspots in GNAQ/11: glutamine(Q)209 and arginine(R)183 (2, 3).

GNAQ and GNA11 encode heterotrimeric G protein alpha subunits of the q class (Gaq/11; ref. 9). They are 90% identical at the amino acid level and play redundant roles; knocking out any three Gnaq and Gna11 alleles is lethal in mice (10). The function of Gaq/11 is to link various 7 transmembrane G protein-coupled receptors (GPCR) to downstream signaling effectors inside cells, canonically phospholipase C (9). Q209 and R183 mutations also activate the MAPK pathway and the Hippo tumor suppressor pathway (2, 3, 11–13). During signaling, GDP-bound alpha subunits are stimulated by ligand binding to GPCRs, which causes the alpha subunits to release GDP, bind GTP, and assume the active conformation that can interact with downstream effectors. Using an intrinsic Ras-like GTP hydrolysis (GTPase) domain, the alpha subunit cleaves the gamma phosphate from the GTP molecule to produce GDP and return the alpha subunit to its inactive conformation (14). Q209 and R183 lie in the GTPase domain and directly position the gamma phosphate for cleavage (15). Substitution mutations at these two highly conserved residues allow for GTP binding, but greatly reduce the rate of GTP hydrolysis, therefore generating constitutive active signaling (16, 17). In uveal melanoma, mutations at Q209 are found 13 times more frequently than mutations at R183 (2). Q209 mutations are also slightly more potent in tumorigenesis assays in nude mice injected with transformed cells (2).

Mutations in GNAQ and GNA11 are found in a striking distribution pattern in different types of melanocytic neoplasms. They are frequently found in uveal melanoma (83%), blue nevi (benign intradermal lesions, 63%), and melanocytomas of the central nervous system (~50%; refs. 2, 18–21). They are very rare among melanomas and nevi located in the epithelium. Initially, we reported a single GNAQ\(^{Q209L}\) mutation among 27 cutaneous melanomas on chronically sun damaged (CSD) skin (3); however, we found no additional mutations in a much larger CSD sample set, nor in any other type of cutaneous melanomas (n = 164; ref. 2). The COSMIC database v72 reports four additional patients with GNAQ\(^{Q209L}\) mutations among 1,696 entries for superficial spreading, lentigo maligna, nodular, and otherwise unspecified malignant melanomas of the skin (22–26). GNAQ and GNA11 mutations have not been found in acral or conjunctival melanoma (2, 3, 27, 28). A GNAQ\(^{Q209P}\) mutation was recently reported in a single case of mucosal melanoma (29).

Intrigued by this pattern, we wondered whether melanocytes in the epithelium possess some natural resistance to the oncogenic
effects of constitutively active GNAQ/GNA11, which could be harnessed for potential therapeutics. To test this, we engineered a flexed-stop GNAQ<sup>Q209L</sup>, conditional knock-in allele at the ubiquitous Rosa26 locus to force expression in these cells, as well as in other types of melanocytes. We report here that the expression of the Rosa26-flexed-stop-GNAQ<sup>Q209L</sup> allele induced by the melanocyte driver, Mitf-cre, causes the rapid development of uveal melanoma, with local invasion of blood vessels and multiple tumors developing in the lungs.

**Materials and Methods**

**Mouse husbandry**

The research described in this article was conducted under the approval of the UBC Animal Care Committee. Strains were crossed to the C3HeB/Fe genetic background for at least 6 generations before use. DNA from ear notches was isolated using DNeasy columns (Qiagen) and amplified using PCR. *Mitf-cre* (Tg (Mitf-cre)7114Gsb), *Tyrosinase-creER* (Tg[Tyr-cre/ERT2]330Bos/)), and *Dct-LacZ* (Tg (Dct-LacZ)A12Jkn) mice were genotyped as previously described (30–32). For intraperitoneal injection, tamoxifen (Sigma T5648) was dissolved in a corn oil/ethanol (10:1) mixture at a concentration of 10 mg/mL. Mice were injected with 1 mg per dose. For topical treatment, tails were dipped in 25 mg/mL 4-hydroxytamoxifen (Sigma H6278) in DMSO.

**Production of Rosa26-flexed-stop-GNAQ<sup>Q209L</sup> mice**

Using pROSA26-1, pSCAlgeo, and PGKknorplox2 plasmids and a human GNAQ<sup>Q209L</sup> cDNA (UMR cDNA Resource Center), a construct was built that contains the minimal adenovirus type 2 major late splice acceptor, a laxP-flanked neo stop cassette, human GNAQ<sup>Q209L</sup>, and a bovine growth hormone polyadenylation signal, all flanked by 1.08-kb and 4.34-kb Rosa26 homology arms, upstream and downstream of the cassette, respectively. The targeting vector was linearized by SacI and then transfected by electroporation of BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem (ES) cells. Following homologous recombination, positive ES cell clones were identified by Southern blotting and PCR and were injected into C57BL/6 blastocysts to produce chimeras, which successfully transmitted the mutant allele to the germline. The PCR reaction components to genotype the Rosa26-flexed-stop-GNAQ<sup>Q209L</sup> allele are 0.25 mmol/L each dNTP, 1 U Hotstar Taq (Qiagen), 1 × Hotstar Taq buffer, and 0.5 μmol/L each primer in 25 μL total volume. The reaction consists of 40 cycles of 95°C for 10 minutes, 58 (1 minute), and 72 (1 minute), using 5′-CCGAAAATCTGTGGGAAGTC and 5′-GGGGCTCTCATGCGTCTCGGAG as primers, which amplify a product of 180 base pairs.

**Histology**

Sections of intact cranium were taken after a 3-day fixation in 10% formalin, followed by decalcification (10% formalin, 88% formic acid). For LacZ analysis, tissue samples were stained for 3-indolyl-b-D-galactopyranoside, and 2.5% dimethylformamide. Immunohistochemistry of sections were stained for lysozyme (Abcam 732, 1:50 dilution) overnight at 4°C, and then incubated for 1 hour at room temperature with Alexa594-conjugated goat anti-mouse antibody (1:500 dilution, Invitrogen). For immunohistochemistry, sections were bleached with 0.5% potassium permanganate for 20 minutes, 2% oxalic acid for 3 minutes, and then incubated in 0.3% hydrogen peroxide for 30 minutes. These sections were blocked with serum containing 1:25 M.O.M. and BSA, incubated with mouse anti-melanoma antibody cocktail (HMB45 + DT101 + BC199; Abcam732, 1:50 dilution) overnight at 4°C, and then incubated 30 minutes with goat-anti-mouse-horseradish peroxidase secondary antibody (1:200, Life Technologies). Sections were washed and stained with DAB for 5 minutes.

For immunofluorescence of β-galactosidase in cultured melanocytes, cells were grown on coverslips, washed with 10 mmol/L sodium phosphate (pH 7.3), fixed in 4% paraformaldehyde for 4°C for 10 minutes, blocked with 1:25 M.O.M. and BSA, incubated with mouse anti-β-galactosidase antibody (1:400, Promega, Z3781) overnight at 4°C, and then incubated for 1 hour at room temperature with Alexa594-conjugated goat anti-mouse antibody (1:500 dilution, Invitrogen).

**Western blotting**

Protein was extracted using the NE-PER Nuclear and Cytoplasmic Extraction kit and Halt Protease inhibitor cocktail (Thermo Scientific) and quantified using the Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich). After protein transfer to nitrocellulose, the membranes were blocked in 3% milk, divided into two sections, and either incubated with 1:1,000 anti-Yap (4912, Cell Signaling Technology) or 1:1,500 anti-GAPDH (14C10, Cell Signaling Technology) for 36 or 40 hours, respectively. Blots were washed two times in PBS for 5 minutes, then incubated with horseradish peroxidase-conjugated goat anti-rabbit (7074, Cell Signaling Technology) at either 1:1,000 or 1:2,000 dilution, respectively, for 3 hours at room temperature. Immunocomplexes were visualized using LumiGlo ECL Prime (GE Healthcare) and a CCD-based imaging system was used to detect immunocomplexes. ImageJ was used to quantify the signal intensity, relative to a specific wild-type sample that was included in every experiment.

**Pyrosequencing**

Flash-frozen uveal melanomas from patients undergoing enucleation as a part of normal patient care were obtained under the approval of the institutional review boards at the University of British Columbia and the Vancouver General Hospital. Primary mouse melanocyte cultures were generated as previously described (33). DNA was extracted using DNeasy columns (Qiagen). RNA was extracted using RNAqueous-4PCR (Ambion), and reverse transcribed using random primers (Superscript VILO, Life Science, Thermo Fisher).

**Immunofluorescence and immunohistochemistry**

Whole eyes were fixed in 4% paraformaldehyde for 1 hour, washed in 8%, 12%, 18%, and 20% sucrose (15 minutes each), incubated in 25% sucrose overnight, embedded in optimal cutting temperature, and sectioned at 8 to 12 μm. For immunofluorescence, sections were blocked with 1:25 mouse on mouse immunoglobulin blocking reagent (M.O.M.; Vector Labs) and BSA, incubated with mouse anti-RPE65 antibody (Abcam 13826, 1:250 dilution) overnight at 4°C, and then incubated for 1 hour at room temperature with Alexa594-conjugated goat anti-mouse antibody (1:500 dilution, Invitrogen). For immunohistochemistry, sections were bleached with 0.5% potassium permanganate for 20 minutes, 2% oxalic acid for 3 minutes, and then incubated in 0.3% hydrogen peroxide for 30 minutes. These sections were blocked with serum containing 1:25 M.O.M. and BSA, incubated with mouse anti-melanoma antibody cocktail (HMB45 + DT101 + BC199; Abcam732, 1:50 dilution) overnight at 4°C, and then incubated 30 minutes with goat-anti-mouse-horseradish peroxidase secondary antibody (1:200, Life Technologies). Sections were washed and stained with DAB for 5 minutes.
Vital Technologies). For PCR, primers were designed to amplify Q209 in both human and mouse GNAQ/Gnaq alleles (Supplemental Materials and Methods). In the first round of PCR, Q209-containing products were amplified, while in the second round, biotin was tagged to the PCR products for subsequent immobilization. PCR reactions consisted of 0.25 mM dNTP, 1 U Hotstar Taq (Qiagen), 1× Hotstar Taq buffer, and 0.5 μM of each primer in 25 μL total volume, with roughly 100 ng of template. Reactions conditions were 95°C (30 seconds), 58.3°C (1 minute), and 72°C (1 minute) for 15 or 20 cycles (first and second rounds, respectively). Pyrosequencing was performed with the PyroMark Q96 MD pyrosequencer (Qiagen; ref. 34).

Auditory brainstem response

Auditory brainstem response tests were performed by the Mouse Biology Program at UC Davis (Davis, CA).

Statistical analysis

Data were analyzed with either ANOVA, Student t test, or Welch two-sample t test.

Results

Creation of the Rosa26-flxed stop-GNAQ<sup>Q209L</sup> allele

We engineered a conditional allele in which GNAQ<sup>Q209L</sup> is expressed from the ubiquitous Rosa26 locus following the removal of a loxp-flanked stop cassette that prevents transcription (35). We built a construct that contains a splice acceptor, a loxp-flanked stop cassette, human GNAQ<sup>Q209L</sup>, and a bovine growth hormone polyadenylation signal (Fig. 1). This allele drives continuous expression of constitutively active GNAQ in cells that undergo Cre-mediated recombination and in all of the descendents of these cells. The resulting Rosa26-flxed stop-GNAQ<sup>Q209L</sup> mice were crossed to the C3HeB/FeJ genetic background for 6 generations before analysis. Rosa26-flxed stop-GNAQ<sup>Q209L</sup> mice are healthy, breed with normal efficiency, and are pigmented normally (Supplementary Fig. S6).

Skin pigmentation changes driven by GNAQ<sup>Q209L</sup> induced by Mitf-cre

To examine the effects of GNAQ<sup>Q209L</sup> expression initiated in melanoblasts (immature melanocytes) during embryogenesis, we obtained Mitf-cre transgenic mice, which express Cre recombinase under the control of the melanocyte-specific promoter of the Microphthalmia gene (31). Mitf-cre is the earliest Cre driver expressed specifically in melanocytes. Mitf-cre has an efficiency of 25% at E15.5 (36) and 68% at P40 (Supplementary Table S1). On the C3HeB/FeJ genetic background, Mitf-cre animals are smaller than nontransgenic animals (Supplementary Fig. S1) and 60% exhibit microphthalmia (n = 35; Supplementary Fig. S4). The cause of these phenotypes is unknown (31). Mitf-cre is expressed in epidermal, dermal, and follicular melanocytes of the tail and trunk skin (31, 36). Expression in uveal, otic, and leptomeningeal melanocytes of the central nervous system (CNS) and spinal cord has not been previously reported.

We crossed Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/+ animals to Mitf-cre/+ animals and obtained the expected percentage of double heterozygous progeny. Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/+; Mitf-cre/+ animals can be distinguished from their littermates 5 days after birth, because they exhibit early stages of tail darkening (Supple-

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A conditional GNAQ^{Q209L} allele. A, the targeted allele contains a splice acceptor, loxP-flanked stop cassette (PGKneo3xpAlox2), GNAQ^{Q209L}, and polyadenylation signal. After loxP recombination, GNAQ^{Q209L} is expressed (activated allele). PCR genotyping amplicon (green). B and C, Southern blot analysis using EcoRI and probing with PB3/4 (B), and EcoRV and probing with AT1/AT2 (C). ES cell clones 114, 132, 133, 242, and 263 are positive. H, HindIII; pA, polyadenylation signal; R, EcoRI; RV, EcoRV; SA, splice acceptor; triangles, loxP sites.
stop-GNAQ$^{Q209L}$/++; Mitf-cre/+ animals (n = 15), but not in +/+; Mitf-cre/+ controls. In younger animals, the uveal tract (composed of the iris, ciliary body, and choroid) was thickened, with a mass forming at the anterior of the eye (Fig. 4A). Older animals exhibited bulging eyes (Supplementary Fig. S4) and bigger tumors that largely filled the vitreous space (Fig. 4C). We performed immunofluorescence using an antibody specific to the retinal pigmented epithelium (RPE; Fig. 4B), and immunohistochemistry with an anti-melanoma cocktail that stains human uveal melanomas (refs. 39, 40; Fig. 4C). The tumors were positive for the anti-melanoma antibody and negative for the RPE antibody. Furthermore, the RPE antibody labeled a single layer of cells above the lesion, with the sclera, the outermost layer of the eye, lying below the lesion. This indicates that the lesions are derived from the uveal tract. By 3 months of age, Rosa26-floxed stop-GNAQ$^{Q209L}$/++; Mitf-cre/+ animals weighed significantly less than

Figure 2.
Melanocyte overgrowth in the dermis and hair follicles of Rosa26-floxed stop-GNAQ$^{Q209L}$/++; Mitf-cre/+ mice. A, hyperpigmentation of ears, footpad, trunk, and tail of 3-month-old Rosa26-floxed stop "fs" -GNAQ$^{Q209L}$/++; Mitf-cre/+ mice (right animal of each subpanel). B, hyperpigmentation of the tail dermis at 3 months of age (hematoxylin and eosin). C, X-gal and eosin staining of Rosa26-floxed stop-GNAQ$^{Q209L}$/++; Dct-LacZ/++; Mitf-cre/+ 5-month-old trunk skin reveals hyperpigmentation of the hair follicles and dermis (white arrowheads) and LacZ-positive cells (blue). D, a dermal lesion (red arrowhead) on the trunk. D, dermis; E, epidermis; HF, hair follicle.
controls, as their overall health was impacted (Supplementary Fig. S1). We conclude that Rosa26-floxed stop-GNAQQ209L drives uveal melanoma with a very short latency period when induced by Mitf-cre.

Human melanocyte cell lines expressing GNAQQ209L exhibit activated YAP within the Hippo tumor suppressor pathway (12, 13). Yap activation can be detected by examining the amount of Yap in the cell nucleus, where it is translocated upon phosphorylation. To examine whether Yap is hyperactivated in uveal melanomas of Rosa26-floxed stop-GNAQQ209L mice, we extracted protein from whole eyes dissected from Rosa26-floxed stop-GNAQQ209L/+; Mitf-cre/+ mice and wild-type controls at 3 months of age. Using Western blotting, we found a single band corresponding to the expected Yap protein size (65–75 kDa). Yap protein was increased 2.5-fold in Rosa26-floxed stop-GNAQQ209L/+; Mitf-cre/+ eyes as compared with wild-type controls (P = 0.02, Welch two-sample t test; Fig. 5A and B). This validates Rosa26-floxed stop-GNAQQ209L mice as a tool for dissecting pathways activated downstream of constitutively active GNAQ.

Expression of GNAQQ209L in mice and humans

To estimate the expression level of Rosa26-floxed stop-GNAQQ209L compared with endogenous Gnaq, we cultured melanocytes from 8.5 day-old mouse tails. We developed primary cultures from the dermis of three different Rosa26-floxed stop-LacZ; Rosa26-floxed stop-GNAQQ209L; Mitf-cre/+ animals. After 2 weeks, we performed immunofluorescence on the cells using a LacZ antibody to detect the percentage of cells expressing GNAQQ209L. We found that 100% of the cells in the cultures were LacZ-positive, likely through an increased growth advantage provided by the GNAQQ209L allele, combined with culture conditions that select for melanocyte growth (Supplementary Fig. S5A; refs. 3, 33).

We then extracted RNA from the cells, reverse transcribed it, and used PCR to amplify the region spanning codon Q209, with primers designed to match both the transgenic human allele and the endogenous mouse alleles, which are highly conserved. The PCR products were pyrosequenced using a single sequencing primer complementary to the forward strand. On average, at the variant position, 97% of the pyrosequencing signal was T (from the endogenous wild-type mouse alleles), while 3% of the signal was A (from the transgenic Rosa26-GNAQQ209L allele; Supplementary Fig. S5). This suggests that there is 16-fold less expression from the Rosa26 promoter than the Gnaq promoter in mice and that full transcription might not be necessary for transformation. Of course, the ideal mouse model would use the endogenous Gnaq promoter, as has been done for BrafV600E (41). However, as explained in the...
Figure 4.
Uveal melanoma in Rosa26-floxed stop-GNAQ^{Q209L/+}; Mitf-cre/+ mice. A, progression of uveal melanoma in 3-week-old (left bottom) and 5-week-old (right bottom) Rosa26-floxed stop-GNAQ^{Q209L/+}; Mitf-cre/+ mice. B, structures of the eye (top). Anti-RPE (red) stains a single layer of retinal pigmented epithelial cells above the developing melanoma in a 3-week-old Rosa26-floxed stop-GNAQ^{Q209L/+}; Mitf-cre/+ mouse (right subpanel; bottom). Corresponding light microscopy images (left and middle). Red box, area of enlargement. C, a uveal melanoma from a 5-month-old mouse stains positive for the antimelanoma antibody cocktail (DAB, brown; top right). Melanoma before bleaching (left) and counterstained with hematoxylin (middle). Red arrow, extraocular extension of the melanoma. Hg, harderian gland; Nc, nasal cavity; red asteriks, uveal melanomas.
Figure 5.
Yap activation and enhanced GNAQ<sup>Q209L</sup> allele expression in uveal melanomas. A, representative Western blot analysis against Yap and Gapdh using nuclear protein extracted from Rosa26-floxed stop-GNAQ<sup>Q209L</sup>/+; Mitf-cre/+ and +/-/+ or Rosa26-floxed stop-GNAQ<sup>Q209L</sup>/+; +/-/+ eyes ("wild-type"). B, increased nuclear Yap is detected in Rosa26-floxed stop-GNAQ<sup>Q209L</sup>/+; Mitf-cre/+ eyes (P = 0.02, Welch two-sample t test). Signal was normalized to Gapdh and a wild-type sample included in each experiment. C, four of five human uveal melanoma samples contain more GNAQ<sup>Q209L</sup> transcript than wild-type transcript, measured by pyrosequencing. Signal is corrected for the amount of each allele in the DNA of each sample. D, bilateral eye bulging (red arrowheads, left) and skin hyperpigmentation (red arrowheads, right) in a 4-month-old tamoxifen-treated Rosa26-floxed stop"fs"-GNAQ<sup>Q209L</sup>/+; Tyr-creER/+ mouse, compared with treated control littermate. E, melanocytic hyperplasia (red arrowhead), but not uveal melanoma, in the eye of the same mouse.
introduction, we were interested in forcing GNAQ<sup>Q209L</sup> expression in melanocytes of the epidermis.

We next asked what relative level of GNAQ<sup>Q209L</sup> to wild-type transcripts is present in human uveal melanomas with a somatic GNAQ<sup>Q209L</sup> mutation. We obtained fresh-frozen uveal melanoma samples removed as a part of normal patient care, extracted DNA and RNA from each sample, reverse transcribed the RNA, and used pyrosequencing to measure the ratio of the GNAQ<sup>Q209L</sup> allele to the wild-type allele in each cDNA sample, relative to the ratio in the corresponding genomic DNA sample. In 4 of 5 uveal melanomas, the GNAQ<sup>Q209L</sup> allele was expressed at a higher level than the wild-type allele, as much as 1.7-fold more (Fig. 5C). Thus, greater levels of GNAQ<sup>Q209L</sup> transcription may provide a selective advantage.

**Tyr-creER** is not sufficient to drive uveal melanoma in mice when initiated in adulthood

To determine whether Rosa26-flxed stop-GNAQ<sup>Q209L</sup> drives uveal melanoma when induced in adulthood, we obtained Tyrosinase(Tyr)-creER mice (32). In this transgene, Cre recombination is fused to the estrogen receptor and requires tamoxifen for the CreER protein to be transported to the nucleus for activity. Tyr-creER expression initiates in melanoblasts at around E12.5 of mouse development (32). To administer tamoxifen, we treated 8-week-old mice with 1 mg tamoxifen by intraperitoneal injection and also dipped the bottom half of the tail in a solution of 25 mg/mL 4-hydroxytamoxifen in DMSO for 5 seconds. This treatment was repeated daily for 5 days. In addition to being absorbed through the skin, topical application of tamoxifen is also known to be consumed by mice during grooming. The efficiency of cre-mediated recombination in epidermal melanocytes of the tail using these combined methods was 39% as determined 7 days following the last dose of tamoxifen (Supplementary Table S3).

Three Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Tyr-creER/+ and 3+/+; Tyr-creER/+ littermates were treated with tamoxifen (as described above) at 8 weeks of age. The Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Tyr-creER/+ and 3+/+; Tyr-creER/+ littermates were treated with tamoxifen as described above at 8 weeks of age (Fig. 5D). Histologic analysis at this time point revealed uniform melanocytic hyperplasia in 4 of 4 tamoxifen-treated Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Tyr-creER/+ eyes. We found the same result in both eyes of one Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Tyr-creER/+ animal aged 6.5 months after treatment (Supplementary Fig. S6B). At the two time points, treated +/+; Tyr-creER/+ and untreated Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Tyr-creER/+ controls were normal (Fig. 5D; Supplementary Fig. S6). We did not judge this melanocytic hyperplasia to be melanoma, because there were no masses extending into the interior of the eye, and the phenotype was stable over time. The bulging eye phenotype might be due to dispersed melanin, which, if it accumulates within the ocular drainage structures, causes elevated intraocular pressure (42). These experiments show that the expression of GNAQ<sup>Q209L</sup> initiated by Tyr-creER in adulthood can promote melanocytic overgrowth in the eye and skin. However, it is not sufficient to drive uveal melanomagenesis within the time frame examined.

**CNS and inner ear overgrowth induced by Mitf-cre**

In addition to the uveal melanomas, we found extensive hyperpigmentation within the cranium of Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/+ mice (n = 28). This was due to melanocytic overgrowth within the leptomeninges (Fig. 6B). We observed invasion of pigmented cells within the orbital-frontal cortex (Fig. 6A) and cerebellum and medulla oblongata (Fig. 6B). In one animal, a large darkly pigmented lesion was also recovered from the brain surface (Fig. 6C). Spinal cord meninges were heavily pigmented in all animals (Supplementary Fig. S7). These findings are consistent with the previously reported presence of GNAQ and GNA11 mutations in human melanocytomas of the CNS (18–21).

The stria vascularis of the cochlea of the inner ear is composed of basal cells, intermediate cells, and marginal cells. Intermediate cells are similar to melanocytes and arise from the neural crest, requiring Pax3, Sox10, c-Kit, and Mitf (43–47). Through Na<sup>+</sup>/K<sup>+</sup>2Cl<sup>–</sup> ion transportation, the stria vascularis produces an endocochlear potential in the endolymph. Changes in the potential in response to sound triggers hair cells to release neurotransmitters that excite afferent nerves. Because we noticed that some older Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/+ animals failed to startle in response to loud noises, we tested the auditory brainstem response (ABR) of 5 Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/+ and animals 5+/+; Mitf-cre/+ control littermates at two time points, P34 and P76. We found that at P76, there is a significant increase in the threshold needed to produce a brainstem response in Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/+ mice compared with +/+; Mitf-cre/+ mice over a broad range of frequencies (ABR click test, Fig. 6D), with individual Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/+ mice worsening in their ability to respond to auditory stimulus with age (Supplementary Table S2).

Melanocyte-like cells are also present in the vestibular system of the inner ear, which regulates balance. Beginning between 1 and 3 months of age, Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/+ mice develop abnormal behaviors that are suggestive of balance deficiencies (e.g., see Supplementary Movie S1). These behaviors include head tossing, head tilting, and flipping onto the back. We performed histologic analysis of the ears at 3 months of age and discovered an extensive overgrowth of pigmented cells, which filled the spaces in the cochlea and vestibular system (Fig. 6E). We note that there were no behavioral abnormalities observed in Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Tyr-creER/+ animals treated with tamoxifen. These experiments indicate that Mitf-cre drives Cre expression in the melanocyte-like cells of the inner ear and that unregulated activity of GNAQ significantly impacts normal inner ear function.

**Evidence of GNAQ<sup>Q209L</sup>-driven metastasis**

Ninety-four percent of the Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/+ mice (n = 19) exhibited at least four pigmented lesions within the lungs at 3 months of age (Fig. 7A). The size of the lung neoplasms ranged from 200 μm to 6 mm in diameter, with 4 to 18 tumors per affected mouse. These tumors could be primary, or be the result of metastasis. While examining the vasculature around the eyes, we found tracks of pigmented cells extending toward blood vessels (Fig. 7C) and invading vessel walls to grow within the lumen (Fig. 7D). An extension of a uveal lesion outside the eye can be seen in Fig. 4C. The lung lesions themselves exhibited blood vessel invasation (Fig. 7E). In addition, heavily pigmented and enlarged lymph nodes were present in every animal in the neck and trunk.
Figure 6.
Melanocytic hyperplasia in the CNS and inner ears. A, hyperpigmentation on the brain surface of a 3-month-old Rosa26-flxed-stop-GNAQ<sup>Q209L/+</sup>; Mitf<sup>cre/+</sup> mouse (middle). Invasion of pigmented cells into the orbital frontal cortex (red asterisk), visible in coronal section (right bottom). B, thickened and hyperpigmented leptomeninges (white arrows in enlargement, top right). Invasion of pigmented cells into the cerebellum and medulla oblongata (white arrowheads in enlargement, right bottom). C, lesion found on the surface of the brain (right). Deep indentation (red arrowhead) left on brain after the lesion was removed. D, hearing loss in Rosa26-flxed-stop-GNAQ<sup>Q209L/+</sup>; Mitf<sup>cre/+</sup> animals at P76 (right), but not P34 (left; mean ± SEM; P = 0.249 at P34; P = 0.044 at P76; ABR click test). E, melanocytic hyperplasia in the cochlea and vestibular system of a Rosa26-flxed-stop-GNAQ<sup>Q209L/+</sup>; Mitf<sup>cre/+</sup> animal (hematoxylin and eosin). CB, cerebellum; Co, cochlea; dB SPL, decibels of sound pressure level; LM, leptomeninges; MO, medulla oblongata; OB, olfactory bulb; OFC, orbital–frontal cortex; V, vestibular system.
Figure 7.
Melanocytic lesions in the lungs and blood vessel intravasation. A, multiple pigmented lesions in the lungs of a 3-month-old Rosa26-flxed stop-GNAQQ209L/++; Mitf-cre/+ mouse (red arrowheads). Front and back of lungs are shown. B, enlarged and darkly pigmented lymph nodes from a 3-month-old Rosa26-flxed stop*fs*-GNAQQ209L/++; Mitf-cre/+ mouse (left). C and D, tracks of pigmented cells extending from eye lesion toward blood vessels (open arrowheads in C), invading vessel walls (black arrow in D), or growing within the lumen (white arrow in D) in 3-month-old Rosa26-flxed stop*fs*-GNAQQ209L/++; Mitf-cre/+ mice (hematoxylin and eosin). Enlarged areas on the right are indicated by white boxes on left. E, lung lesion closely associated with blood vessels showing signs of intravasation (black arrow) in a 3-month-old Rosa26-flxed stop*fs*-GNAQQ209L/++; Mitf-cre/+ mouse (hematoxylin and eosin). BV, blood vessel.
These data indicate that GNAQ<sup>Q209L</sup> expression promotes melanoma metastasis.

**Discussion**

Gnaq and Gna11 were first identified as melanocyte regulators during a forward genetic screen of darker skinned mice (48, 49). Hyperactive, but not constitutively active, germline mutations in Gnaq and Gna11 were identified in three independent mouse mutants with a darker dermis and a normal epidermis. Similarly, somatic mutations in GNAQ and GNA11 have been frequently found in melanoma and nevi located in the dermis, but are rare in human melanoma and nevi that are located in the epithelium (2). It is not known why lesions located in the epidermis and dermis exhibit different mutational signatures, although there are several possibilities. First, there could be intrinsic or developmental differences among melanocytes colonizing different parts of the skin (36, 50). Second, there could be different paracrine signals emanating from keratinocytes versus fibroblasts, surrounding melanocytes in the epidermis and dermis, respectively. Third, melanocytes in the dermis and epidermis could be exposed to different types and/or intensities of environmental mutagens, such as UV light.

We were curious as to whether the absence of GNAQ<sup>Q209L</sup> mutations in lesions located in the epithelium was a result of a lack of expression of GNAQ or a differential response downstream of it. To test this, we used the ubiquitous Rosa26 promoter to force expression of Gnaq and Gna11 (Fig. 7B). These data indicate that Huang et al. downstream effects of Braf oncogenic for uveal melanocytes, but is far less so for dermal interfollicular epidermis. Furthermore, GNAQ<sup>Q209L</sup> promotes melanoma metastasis.

Ther eol of GNAQQ209L appears to be highly conserved in other species (37, 41, 51–53). The dermal location of GNAQ/11 mutations is dominant of melanocyte cell fate(54), while Tyrosinase, a melanogenic enzyme, is expressed only in differentiated melanocytes (55). Therefore, Mitf might be expressed in cells with a higher potential for transformation. Recently, the promoter of another melanogenic enzyme, Dopachrome tautomerase (Dct), was used to drive transgenic GNAQ<sup>Q209L</sup> expression in a tetra-cycline-inducible mouse model (13). Despite expression beginning during embryogenesis, no ocular or skin lesions developed. Pigmentation could not be assessed because the mice were on the FVB genetic background, which is albino. When the tumor suppressors, p16 and p19, were also knocked out, 50% of these mice developed lesions on the trunk by 9 months of age, but there were still no reported effects on the eye. Thus, Mitf-cre may better target uveal melanocytes or melanocytes with a higher melanomagenic capacity than Tycr-cre or Dct-cre. Another intriguing possibility that remains to be tested is whether the Mitf-cre BAC transgene somehow alters Mitf protein levels, which could theoretically contribute to melanogenesis (56).

In summary, we have developed the first mouse model of uveal melanoma driven by oncogenic GNAQ. The three known human lesions driven by GNAQ/11 mutations are recapitulated in the mice (blue nevi, uveal melanoma, and melanocytomas of the CNS). We have established that GNAQ<sup>Q209L</sup> has multiple effects depending upon the location of the melanocytes. The rapid localized invasion of blood vessels and multiple lung tumors by 3 months of age suggests that GNAQ<sup>Q209L</sup> mutations may not only help initiate uveal melanoma, as previously hypothesized, but also contribute to disease progression.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: J.L.-Y. Huang, C.D. Van Raamsdonk Development of methodology: J.L.-Y. Huang, C.D. Van Raamsdonk Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Urtatiz, C.D. Van Raamsdonk Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L.-Y. Huang, O. Urtatiz, C.D. Van Raamsdonk Writing, review, and/or revision of the manuscript: J.L.-Y. Huang, O. Urtatiz, C.D. Van Raamsdonk Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L.-Y. Huang Study supervision: C.D. Van Raamsdonk

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References


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