The hyperparathyroidism-jaw tumour (HPT-JT) syndrome is an autosomal dominant disorder characterized by occurrence of parathyroid tumours, often atypical adenomas and carcinomas, ossifying jaw fibromas, renal tumours and uterine benign and malignant neoplasms. HPT-JT is caused by mutations of the cell division cycle 73 (CDC73) gene, located on chromosome 1q31.2 and encodes a 531 amino acid protein, parafibromin. To facilitate in vivo studies of Cdc73 in tumourigenesis we generated conventional (Cdc73+/−) and conditional parathyroid-specific (Cdc73+/+−/PTH-Cre and Cdc73+−/−/PTH-Cre) mouse models. Mice were aged 18-21 months and studied for survival, tumour development and proliferation, and serum biochemistry, and compared to age-matched wild-type (Cdc73+/+ and Cdc73+−/−/PTH-Cre) littermates. Survival of Cdc73+−/− mice, when compared to Cdc73+−/− mice was reduced (Cdc73+−/− = 80%; Cdc73+−/− = 90% at 18 months of age, P < 0.05). Cdc73+−/− mice had significantly increased proliferation, with rates >fourfold higher than that in parathyroid glands of wild-type littermates (P < 0.0001). Cdc73+−/−, Cdc73+−/−/PTH-Cre and Cdc73+−/−/PTH-Cre mice also had increased mean serum parathyroid hormone (PTH) concentrations. Parathyroid tumour development, and elevations in serum calcium and PTH, were similar in males and females. Cdc73+−/− mice did not develop bone or renal tumours but female Cdc73+−/− mice, at 18 months of age, had uterine neoplasms comprising squamous metaplasia, adenofibroma and adenomyoma. Uterine neoplasms, myometria and jaw bones of Cdc73+−/− mice had increased proliferation rates that were 2-fold higher than in Cdc73+−/− mice (P < 0.05). Thus, our studies, which have established mouse models for parathyroid tumours and uterine neoplasms in the HPT-JT syndrome, provide in vivo models for future studies of these tumours.
animals obtained by backcrossing onto wild-type C57BL/6 females for ten generations. Expression of wild-type and mutant Cdc73 and parafibromin, was detected by RT-PCR (Figure 1b) and western blot analysis (Figure 1c), respectively, to establish the wild-type (Cdc73+/+), and heterozygote (Cdc73+/+/PTH-Cre) referred to as Cdc73p/+ genotypes of adult mice. Cdc73−/− mice were viable and fertile and homozygote (Cdc73−/−/−) referred to as Cdc73−/− mice have been previously reported to demonstrate embryonic lethality.19 Kaplan–Meier analysis of 284 mice, comprising 104 Cdc73−/− mice (n = 36 male, 68 female) and 180 Cdc73+/− mice (n = 72 male, 108 female) aged to 18 months, revealed a significantly decreased survival of Cdc73−/− mice compared to Cdc73+/− mice (survival of Cdc73−/− versus Cdc73+/− mice = 80% versus 90%, Figure 2a, P < 0.05). Further analysis of this data by gender, revealed that the decreased survival of Cdc73−/− mice was largely due to decreased survival of male Cdc73−/− mice, which was observed from 7 months of age (Figure 2b); the survival of Cdc73−/− male and female mice was similar (Figure 2c). The decreased survival in male Cdc73−/− mice was not associated with lower body weight (Figure 2d), which was consistent with the reported mean body weight for C57BL/6 mice of similar ages.23

Parathyroid-specific Cdc73 conditional knockout mice, were generated by mating parathyroid hormone (PTH)-Cre transgenic mice25 with previously established Cdc73−/− mice.26 This resulted in mice deleted for one or both Cdc73 alleles in the parathyroids, that yielded heterozygote Cdc73−/−/PTH-Cre and homozygote Cdc73−/−/PTH-Cre mice, respectively,19,24 which were viable and fertile. A total of 52 parathyroid-specific Cdc73 knockout mice were generated, and comprised 20 Cdc73−/−/PTH-Cre mice, 15 Cdc73−/−/PTH-Cre mice and 17 Cdc73+/−/PTH-Cre mice.

Examination for development of HPT-JT associated tumours. Development of HPT-JT associated tumours was assessed in 69 mice (21 Cdc73−/− mice (9 males and 12 females) and 48 Cdc73+/− mice (20 males and 28 females)) between the ages of 7 to < 24 months (Table 1). Parathyroid tumours were found to occur in 68% of Cdc73−/− mice, and 25% of these were adenomas and 75% were APAs (defined by having collagenous fibrous septa,28 and/or immunostaining for galectin-3 but lacking evidence of invasion or metastasis). We used immunostaining for galectin-3, which is an anti-apoptotic lectin that regulates cyclin D1 and C-Jun N-terminal kinase 1 (JNK1) expression and promotes tumour growth and metastasis.20,27 as it has been reported to have a sensitivity of > 95% and specificity of 90% for pathological diagnosis of PC in man.29 Uterine neoplasms were found in ~ 33% of Cdc73−/− females (Table 1). Parathyroid tumours or uterine neoplasms were not found to occur in Cdc73−/− mice. Ossifying fibromas of the jaw, and tumours of the kidneys, thyroid, pancreas or testis (Table 1) were not found in any of the Cdc73+/− or Cdc73−/− mice. Parathyroid tumour development was also found to occur in ~ 40% of the 32 conditional knockout mice, which comprised 15 Cdc73−/−/PTH-Cre mice (9 males and 6 females) and 17 Cdc73−/−/PTH-Cre mice (8 males and 9 females), but not in any of 20 Cdc73+/−/PTH-Cre mice (11 males and 9 females), aged 20–21 months of age. The parathyroid and uterine neoplasms developing in the mutant mice were further studied.

Analysis of parathyroid tumours Parathyroid glands were identified in 74% of mice (n = 109) and the remaining 26% of mice in whom parathyroid glands were not identified were evenly distributed among all the genotypes. Parathyroid tumours were found in > 65% of Cdc73−/− mice (n = 15/22) but in none of 16 Cdc73+/− littermates (P < 0.0001, two-tailed Fisher’s exact test, Table 1), between 9 and 18 months of age; and in ≥ 50% of ≥ 18-month-old Cdc73+/−/PTH-Cre (n = 7/12)

Table 1. Proportion of tumours (percent) in patients with the hyperparathyroidism-jaw tumour (HPT-JT) syndrome and Cdc73−/− mice

| Tumour | HPT-JT patients | Cdc73−/− mice (> 18 months) |
|--------|-----------------|-----------------------------|
| Parathyroid | 82% adenoma (108/132) | 68% overall |
| 15% carcinoma (20/132) | 25% adenoma |
| 75% atypical adenoma |
| Mandible | 33% ossifying fibroma (67/205) | 0% |
| Uterus | 74% overall (20/27) | 33% overall |
| 53% adenomyosis (8/15) | 100% endometrial cysts |
| 33% adenofibroma (5/15) | 25% endometrial hyperplasia |
| 27% endometrial hyperplasia (4/15) | 13% adenofibroma |
| 27% leiomyoma (4/15) | 13% adenomyoma |
| Kidney | 13% adenocarcinoma (2/15) | 0% |
| Thyroid | < 2% Papillary thyroid carcinoma (2) | 0% |
| < 1% Hurler cell adenoma (1) |
| Pancreas | < 1% Adenocarcinoma (1) | 0% |
| < 1% Mixed germ cell tumour (1) |

sequence identity and 47% similarity to the yeast Cdc73 protein, which is a component of the polymerase-associated factor-1 (Paf1) complex,13 a key transcriptional regulatory complex that interacts directly with RNA polymerase II. The crystal structure of the yeast Cdc73 C-domain has been reported to adopt a Ras-like fold that participates in histone ubiquitination and methylation steps through both promoter and coding regions, and studies have shown that human homologues of the yeast Paf1 complex are associated with parafibromin.14–16 Moreover, parafibromin and its Drosophila homologue, Hyrax, which is a component of the Wnt1 wingless pathway and has an essential role in normal embryonic development, have a high degree of sequence similarity in their C-terminal portions.17,18 This suggested that parafibromin may have a role in embryonic development and studies of mice deleted for Cdc73 have also shown that parafibromin has key roles in mammalian embryonic development.19 Thus, Cdc73 null mice were embryonic lethal by 6.5 day post-coitum, which is the stage when implantation occurs.19 Parafibromin and Hyrax also have a high degree of sequence similarity in their N-terminal domains, which directly interact with β-catenin/Armadillo in the context of the PAF1 complex.17 The role of parafibromin as a mediator of Wnt signalling is supported by studies in human HEK293 cells, which has shown that Wnt target gene expression is directly correlated with parafibromin expression.17 Moreover, parafibromin overexpression in HEK293 and NIH3T3 cells strongly inhibits proliferation, and in HeLa cells it increases G1 phase arrest and apoptosis with a concomitant reduction in S-phase entry, and a resulting downregulation of the cell cycle regulator cyclin D1, which is an oncogene known to be upregulated in parathyroid tumours.19–21 Furthermore, underexpression of parafibromin, induced by RNAi, has been reported to increase the proportion of HeLa cells in S-phase, and to reduce basal apoptosis.15 These in vitro findings indicate that parafibromin is a likely tumour suppressor in mammalian cells, and to explore further the role of parafibromin as an in vivo tumour suppressor we studied mice deleted for Cdc73 for the development of tumours.

RESULTS

Generation, viability and survival of mice deleted for Cdc73 alleles

Conventional Cdc73 knockout mice were established using the embryonic stem (ES) cell line (RRE190) from Bay Genomics Genetrap resource (Figure 1a),22 as described,19 and congeneric
and Cdc73+/−/PTH-Cre (n = 6/12) mice, but not in any of 19 Cdc73+−/PTH-Cre littermates (P < 0.005). The parathyroids in wild-type mice were ~500 μm in length (Supplementary Figure 2) and had a homogenous appearance (Figure 3a). Parathyroid tumours (Figures 3b–d) and which were ~1 mm in length (Supplementary Figure 2) and had a heterogeneous architecture, developed in Cdc73+/−, Cdc73−+/−/PTH-Cre and Cdc73−+/−/PTH-Cre mice, and these demonstrated abnormalities that included glandular enlargement, nuclear pleomorphism, and septation (Figures 3b–d), which are features often observed in PCs and APAs. Indeed 75% of the parathyroid tumours of Cdc73+/−/PTH-Cre and Cdc73−+/−/PTH-Cre mice, when compared to wild-type littermates had features found in APAs (Table 1),9 which included: increased collagen deposition in the septa (Figures 3e–h); reduced nuclear expression of parafibromin (Figures 3i–l); and increased expression of galectin-3 (Figures 3m–p). Loss of
from Cdc73+/−, Cdc73+/L−/− mice (data not shown). To assess the proliferation rate of these parathyroid tumours, mice were given the thymidine analogue BrdU in drinking water for two weeks, and the proportion of cells that had incorporated nuclear BrdU was calculated (Figures 3q–t, Table 2). The parathyroid tumours developing in the Cdc73+/− and Cdc73+/+ /PTH-Cre mice had significantly higher daily proliferation rates, by three- to fourfold, while that of Cdc73−/−/PTH-Cre mice was ∼9-fold higher, when compared to those of parathyroid glands in wild-type mice Cdc73+/+ and Cdc73−/+ /PTH-Cre mice (Table 2, P < 0.0001). Apoptotic rates in parathyroids of Cdc73+/+ , Cdc73−/+ /PTH-Cre and Cdc73−/−/PTH-Cre mice were not significantly different from wild-type (Cdc73+/+ and Cdc73−/+ /PTH-Cre) littermates (Figures 3u–x, Supplementary Figure 4).

Parathyroid tumours in the Cdc73+/− (n = 25, age > 17 months), when compared to similarly aged wild-type Cdc73+/+ littermates (n = 20), were associated with increased mean (± s.e.m.) serum calcium concentrations (Cdc73+/− versus Cdc73+/+ = 2.85 ± 0.04 mmol/l versus 2.66 ± 0.08 mmol/l, P < 0.05, Figure 4a), that was accompanied by a significantly increased mean (± s.e.m.) serum parathyroid hormone (PTH) concentration (Cdc73+/− versus Cdc73+/+ = 81.34 ± 10.29 pmol/l versus 52.03 ± 7.43 pmol/l, P < 0.05, Figure 4b). The serum phosphate (Figure 4c), creatinine (data not shown) and albumin concentrations (data not shown) were not statistically different between Cdc73+/− and Cdc73+/+ mice aged 17–24 months, and Cdc73+/− and Cdc73+/+ mice ≤12 months of age had no statistical differences in serum calcium, adjusted for albumin, or serum phosphate concentrations (data not shown). Thus, Cdc73+/− mice over 17 months of age had features of primary hyperparathyroidism. Cdc73−/−/PTH-Cre (n = 5, age > 20 months) and Cdc73−/−/PTH-Cre (n = 5, age > 20 months) mice, when compared to Cdc73+/−/PTH-Cre (n = 9) littermates also had elevated mean (± SEM) serum calcium concentrations (Cdc73−/−/PTH-Cre = 2.81 ± 0.07 mmol/l, Cdc73−/−/PTH-Cre = 2.76 ± 0.08 mmol/l and Cdc73−/−/PTH-Cre = 2.52 ± 0.07 mmol/l, P < 0.01). The serum albumin (data not shown), creatinine (data not shown) and phosphate concentrations were not significantly different (Cdc73−/−/PTH-Cre = 3.27 ± 0.13 mmol/l, Cdc73−/−/PTH-Cre = 3.37 ± 0.40 mmol/l and Cdc73−/−/PTH-Cre = 2.99 ± 0.23 mmol/l).

Analysis of uterine neoplasms
Macroscopic uterine tumours at necropsy were observed in 33.3% (n = 8/24) of female Cdc73+/− mice, aged ≥18 months, but in none of 24 Cdc73+/+ littermates (P < 0.005, two-tailed Fisher’s exact test). Histology of ≥18 month old Cdc73+/− mice demonstrated several abnormalities, when compared to those from Cdc73+/+ littermates (Figures 5a–e). Thus, uteri from Cdc73+/− mice had glandular endometria with a uniform mucosal epithelium, whereas uteri from Cdc73+/− mice had: large cysts within the...
endometrium (Figures 5b–e); endometrial hyperplasia with areas of squamous metaplasia (Figures 5b–c); and bridging of the endometrial lining across the uterine lumen (Figures 5b–e). Furthermore, 

Cdc73+/- mice had uterine tumours which included an adenofibroma (Figure 5d) and an adenomyoma (Figure 5e), that were not observed in Cdc73+/+ littermates. Parafibromin expression was present in uteri of ≥18 month old Cdc73+/- mice (Figure 5f), but was reduced in uterine tumours (for example, fibroadenoma, Figure 5g) of Cdc73+/+ mice. Moreover assessment of progestosterone receptor expression, which is a favourable prognostic marker in uterine tumours,26–35 revealed strong endometrial expression of progestosterone receptor in uteri of Cdc73+/- mice (Figure 5h), but absent endometrial progestosterone receptor expression in all of the hyperplastic, fibroadenoma and adenomyomas from Cdc73+/+ mice (Figure 5i), which instead had increased stromal expression of progestosterone receptor.

Assessment of proliferation rates, using BrdU incorporation, revealed the myometria from Cdc73+/- mice to have a ~2-fold increase in proliferation rates when compared to that of wild-type littermates (Table 2, P < 0.05). This was confirmed by immunostaining for Ki-67, which revealed a significantly increased proliferation in the endometria and myometria of Cdc73+/- mice with uterine tumours, by 1.5- and 2.5-fold, respectively, when compared to equivalent uterine tissues of wild-type littermates (Figures 5j–l).

Other tumours

Tumours of the bones, kidneys, thyroid, pancreas, or testes, which occur in HPT-JT respectively, were rare (or not found) in Cdc73+/- mice, but were not found in Cdc73+/+ mice. The basis of these inter-species differences remain to be defined but they may be partly due to: the methods of detection, which may have missed detecting the small tumours in the Cdc73+/- mice; the possible later onset of tumours, as suggested by the finding of the increased mandibular cell proliferation rate which may represent a pre-malignant or early neoplastic phase of tumourigenesis; the possible functional redundancy of parafibromin for tumourigenesis in kidneys, pancreas, testes and jaw of mice; and the effects of species-specific genetic modifiers that might alter the phenotypic expression of the Cdc73 mutation in a species. However, it is important to note that the Cdc73+/- mice developed two of the most common tumours, namely parathyroid tumours and uterine neoplasms that are observed in HPT-JT patients. Thus, Cdc73+/- mice provide an in vivo model for the study of APAs and uterine neoplasms. The development of these tumours occurred in Cdc73+/- mice having a reduced survival (Figure 2a). Moreover, survival of Cdc73+/- males was significantly less than Cdc73+/- females (Figure 2b), even though there were no differences in mean serum calcium or PTH concentrations, or the development of parathyroid tumours, between the genders. The basis of the decreased survival in Cdc73+/- males remains unknown, but a reduction in signalling via insulin-like growth factor-1 (IGF-1), which is reported to favour female longevity in mice,37,38 may be contributing, especially as parafibromin in murine embryonic fibroblasts has been reported to bind the IGF-1 promoter, and the loss of parafibromin has been observed to decrease expression of IGF-1.39 Finally, our results, which demonstrate that both conventional (Cdc73+/−) and conditional (Cdc73+/−/PTH-Cre and Cdc73+/−/PTH-Cre) knockout mice develop parathyroid tumours, indicate that Cdc73 has a critical role in parathyroid tumourigenesis. PCs, which have an incidence from 0.5% to 5% of primary hyperparathyroidism cases, may metastasize to regional lymph nodes or distant sites such as lungs, liver, bone or pancreas, and patients will generally die from complications of the associated hypercalcaemia.39 The only curative treatment for PC is en bloc resection of the primary tumour.2 However, PC cannot be easily distinguished from APA or PA pre-operatively or intra-operatively, in the absence of macroscopic tumour invasion or metastasis, and thus most patients with PC do not receive curative surgery and require long-term medical management.3,9 Medical therapies, including medical therapy and radiotherapy, are ineffective with the exception of cinacalcet, an allosteric modulator of the calcium-sensing receptor, which is effective in correcting the hypercalcaemia.39 Thus, improved medical therapies for PC are required. Parafibromin immunostaining may represent an important prognostic marker, as loss of parafibromin immunostaining has been reported to be associated with decreased disease-free survival and tumour recurrence in patients with PCs.30,41 Moreover, APAs with loss of parafibromin immunostaining are considered tumours of uncertain malignant potential as their recurrence rate is higher at 20% when compared to a 0% recurrence rate in APAs that express parafibromin.30,41 Thus, our conventional and conditional Cdc73 knockout mouse models that develop APAs lacking parafibromin expression will facilitate studies aimed at understanding the molecular pathogenesis of APAs, PCs and PAs, and in providing pre-clinical models for evaluating drugs.

Cdc73 mutations occur in ~70% of patients with sporadic, non-syndromic PCs, and in >75% of patients with HPT-JT. Indeed, Cdc73 abnormalities, either due to mutations or LOH are the major driver for PCs in humans, although expression of a mutated parafibromin protein rather than complete loss of parafibromin expression has also been reported in some PCs.2 In addition copy number gain of mutant Cdc73 alleles, with loss of the wild-type
*CDC73* allele through focal deletion or loss of the chromosomal arm have also been reported, and the roles and mechanisms of such selections of mutated *CDC73* alleles in parathyroid tumourigenesis remains to be elucidated. Mutations involving other genes are rare, and to date 6 multiple endocrine neoplasia type 1 (*MEN1*) germline mutations and 2 rearranged during transfection (*RET*) germline mutations have been reported in patients with PCs occurring in association with *MEN1* and *MEN2A*, respectively.

![Image of histological sections showing different staining techniques](image-url)
and 5 Prune Homolog 2 (PRUN2) mutations (1 germline and 4 somatic) have been reported in PCs. Other genetic abnormalities that have been detected in human PCs include: retinoblastoma (RB) loss of heterozygocity (LOH) and loss of expression (LOE) in >85% of PCs;46 cyclin D1 (CCND1) overexpression in >90% of PCs;30 adenomatous polyposis coli (APC) LOH and LOE in ~75% of PCs;47 tumour protein 53 (TP53) LOH and LOE in 33% of PCs;48 glycolgen synthase kinase 3β (GSK3β) LOE in 33% of PCs;47 and enhancer of zeste homolog 2 (EZH2) gene amplification in 60% of PCs.49 Abnormalities of these genes are not necessarily associated with PCs in mice. For example Men1+/− mice develop PAs but not carcinomas;50 transgenic mice overexpressing cyclin D1 develop adenomas but not carcinomas;51 Rb+/− mice develop medullary thyroid carcinomas and pituitary adenocarcinomas but not PCs;52 and Men1+/−/Rb+/− mice developed pituitary, thyroid and pancreatic islet hyperplasia, but not PCs.52 These findings indicate that loss of RB expression and increase of cyclin D1 expression may not be required for PC development in the mouse, and are consistent with our observations that RB and cyclin D1 expression were not altered in the APAs of Cdc73+/− mice. Moreover, these findings indicate that Cdc73 abnormalities represent the major driver for PCs in humans and APAs in mice.

Uterine corpus tumours, are common, occurring in >30% of women >40 years, and may be benign or malignant.53 Uterine tumours may originate from: the epithelial layer for example, endometrial hyperplasia or carcinoma; the mesenchymal layers,

| Tissue | Genotype | Mean proliferation ratea | Fold change vs wild type | P-value vs Cdc73+/− |
|--------|----------|--------------------------|--------------------------|-------------------|
| Parathyroid | Cdc73+/− | 0.150 ± 0.020 | — | — |
| | Cdc73−/− | 0.628 ± 0.101 | 4.2 | 0.0001 |
| | Cdc73+/−/PTH-Cre | 0.165 ± 0.016 | — | 0.380 |
| | Cdc73−/−/PTH-Cre | 0.513 ± 0.053 | 3.1 | <0.0001 |
| | Cdc73+/−/PTH-Cre | 1.416 ± 0.389 | 8.6 | <0.0001 |
| Mandible | Cdc73+/− | 0.653 ± 0.170 | — | — |
| | Cdc73−/− | 1.476 ± 0.254 | 2.3 | 0.014 |
| Uterus | Myometrium | Cdc73+/− | 0.526 ± 0.063 | 1.7 | 0.046 |
| | Cdc73−/− | 0.900 ± 0.168 | — | — |
| Endometrium | Cdc73+/− | 1.903 ± 0.244 | 1.0 | 0.924 |
| | Cdc73−/− | 1.932 ± 0.175 | — | — |
| Kidney | Cdc73+/− | 0.371 ± 0.023 | — | — |
| | Cdc73−/− | 0.374 ± 0.023 | 1.0 | 0.925 |
| Pancreas | Exocrine | Cdc73+/− | 0.208 ± 0.032 | — | — |
| | Cdc73−/− | 0.293 ± 0.046 | 1.4 | 0.136 |
| Endocrine | Cdc73+/− | 0.363 ± 0.045 | — | — |
| | Cdc73−/− | 0.398 ± 0.033 | 1.1 | 0.533 |

Abbreviation: cdc73, cell division cycle 73. *A minimum of four animals per genotype and a minimum of four sections per animal were analysed.*

**Figure 3.** Parathyroid tumours develop in Cdc73+/− and parathyroid-specific Cdc73+/−/PTH-Cre and Cdc73+/−/PTH-Cre knockout mice. (a–d) H&E-stained sections of parathyroid glands from wild-type (Cdc73+/+), heterozygote (Cdc73+/− and Cdc73+/−/PTH-Cre), and homozygote (Cdc73+/−/PTH-Cre) mice, showing: (a) homogenous histology of a wild-type parathyroid; (b) enlarged PA from a Cdc73+/− mouse; (c) a large PA from a Cdc73+/−/PTH-Cre mouse, with increased septation and irregular outline; and (d) abnormal architecture of a parathyroid gland from a Cdc73+/−/PTH-Cre mouse, with increased lipid deposition, nodularity, necrosis and septation. (E–H) Masson’s trichrome stained sections of parathyroids of each genotype demonstrating collagen (blue), and increased fibrous septation in Cdc73+/−, Cdc73+/−/PTH-Cre and Cdc73+/−/PTH-Cre mice when compared to Cdc73+/+ mice. (i–l) Nuclear paraffinomembrin protein expression (brown) in parathyroids was absent or reduced in Cdc73+/−, Cdc73+/−/PTH-Cre and Cdc73+/−/PTH-Cre mice, thereby confirming the parathyroid-specific loss of Cdc73 expression resulting from the presence of PTH-Cre (Supplementary Figure 3). (m–p) Galectin-3 protein expression (brown cytoplasm) in parathyroids was increased in Cdc73+/−, Cdc73+/−/PTH-Cre and Cdc73+/−/PTH-Cre mice (m–p), when compared to Cdc73+/+ mice (m). (q–t) Assessment of parathyroid tumour proliferation by immunofluorescent BrdU incorporation, by continuous administration of BrdU in drinking water, showing that: (q) few parathyroid cells had proliferated in Cdc73+/− mice; but that (r–t) higher proportions of parathyroid cell nuclei had incorporated BrdU in the parathyroid tumours of Cdc73+/−, Cdc73+/−/PTH-Cre and Cdc73+/−/PTH-Cre mice. (s) A rim of normal parathyroid tissue, to the left of the image, had low proliferation, whilst the tumour nodule demonstrated focal areas with a high proportion of nuclei that had incorporated BrdU. BrdU-containing nuclei (red, arrows) indicate cellular proliferation; DAPI nuclear counterstain (blue). (u–x) Assessment of apoptosis by TUNEL assay. Apoptotic cells (green nuclei, arrows) were infrequently observed in parathyroids from mice of each genotype; DAPI nuclear counterstain (blue). Scale bars represent 200 μm; insets have x400 magnification.
for example, leiomyomas (uterine fibroids), which are benign smooth muscle tumours that develop in the myometrium; or both (that is, mixed epithelial and mesenchymal) layers, for example, carcinomas which have malignant epithelial and mesenchymal components, and adenocarcinomas which are neoplasms composed of benign epithelium but malignant stroma. The uterine tumours that develop in women with HPT-JT include benign tumours such as endometrial hyperplasia, adenomyosis, adenofibromas and leiomyosis, and malignant tumours, such as adenocarcinomas. Cdc73+/− female mice developed uterine tumours, that were representative of those in women with HPT-JT and these included endometrial hyperplasia, adenomyoma and adenofibroma. These uterine neoplasms developed in ~33% of female Cdc73+/− mice (Figure 5), whilst spontaneous uterine lesions were not observed in wild-type mice in our study and are also reported to be exceedingly rare in normal wild-type mice. Thus, these Cdc73+/− female mice provide a model to investigate the molecular basis of uterine tumourigenesis. A previous study of human uterine tumourigenesis has reported >70% of Mullerian adenocarcinomas to have: copy number gain for MYB proto-oncogene like 1 (MYBL1), mouse double minute 2 proto-oncogene (MDM2) and cyclin dependent kinase 4 (CDK4); copy number loss for cyclin dependent kinase inhibitor 2A (CDKN2A), breast cancer type 1 susceptibility protein (BRCA1)-associated protein 1 (BAP1) and RB1; single nucleotide variations including nonsense mutations for TP53 and alpha thalassemia/mental retardation syndrome X-linked (ATRX); and mutations in signalling pathways notably PI3K-AKT/PTEN. In addition, >90% of leiomyomas (fibroids) have upregulation of G-protein coupled receptor 10 (GPR10) resulting in activation of the PI3K/AKT-mTOR pathway; while 70% of leiomyomas have a mutation of the mediator complex subunit (MED12) gene that encodes a scaffold protein which interacts with proteins that include β-catenin. It is interesting to note that parafibromin also directly interacts with β-catenin in the PAF complex to mediate Wnt signalling, whose dysregulation has been reported to be associated with development of intestinal and colon cancers, and it may be that similar pathways are involved in uterine tumourigenesis. Analysis of mouse embryonic fibroblasts from Cdc73+/− and Cdc73−/− mice revealed that the parafibromin/PAF complex regulated genes involved in cell growth and survival including the chromatin remodelling genes high mobility group AT-hook 1 (Hmga1) and 2 (Hmga2) to which parafibromin and PAF directly bind. Moreover, parafibromin may also act indirectly via HMGA1 which is a downstream mediator of aberrant Wnt signalling. Thus, loss of parafibromin expression in the mouse embryonic fibroblasts of Cdc73−/− mice has been reported to lead to downregulation of Hmga1. However, this role of parafibromin in uterine tumourigenesis requires cautious extrapolation, as Hmga1 overexpression in transgenic female mice with 1-28 copies of Hmga1a, is associated with development of uterine tumours resembling human uterine adenocarcinomas. Finally, Wilms Tumour 1 protein (WT-1), which is often expressed in Mullerian adenocarcinoma, has been reported to bind to the Cdc73 promoter and to repress Cdc73 expression in oral squamous cell carcinoma. The roles of these interactions of parafibromin in the aetiology of uterine tumourigenesis remain to be explored and our understanding of the Cdc73−/− mouse, which develop uterine tumours, will help to provide an important resource in these studies.}

In summary, we have established a conventional Cdc73−/− mouse, in which males and females develop PAs and APAs, and females develop uterine tumours; thus this Cdc73−/− mouse model is representative of the human HPT-JT syndrome. Moreover, we have developed parathyroid-specific Cdc73 knockout mouse models, which develop APAs and PAs. These mouse models will facilitate further in vivo investigations of the role of parafibromin in parathyroid and uterine tumourigenesis.

**Figure 4.** Cdc73−/− mice have increased mean serum calcium and PTH concentrations, when compared to Cdc73+/− mice. (a) Serum calcium concentration, adjusted for albumin concentration, revealed an increased mean serum calcium concentration in Cdc73−/− mice with parathyroid tumours when compared to Cdc73+/− littermates with normal parathyroids (P < 0.05). (b) Mean serum PTH concentration was elevated in Cdc73−/− mice with parathyroid tumours compared to Cdc73+/− littermates with normal parathyroids (P < 0.05). (c) Serum phosphate concentration in Cdc73−/− mice with parathyroid tumours compared to Cdc73+/− littersmates with normal parathyroids were as shown as filled symbols and Cdc73−/− littersmates with normal parathyroids are shown as open symbols. Squares represent males and circles represent females. The age range of the mice was 17–24 months (mean ± s.e.m. = 20.0 ± 0.30). Combined results from males and females for serum calcium, phosphate and PTH concentrations are shown, as there were no significant gender differences. Horizontal lines indicate mean values together with the standard error of the mean (s.e.m.), which is shown numerically below each group and the number (N) of mice.
Figure 5. Uterine abnormalities develop in Cdc73+/− mice. (a) H&E-stained section of a uterus from a Cdc73+/+ mouse showing a normal myometrium (Myo), endometrium (Endo) and central lumen (l). (b, c) H&E-stained sections of uteri from Cdc73+− mice with endometrial hyperplasia (b) and endometrial hyperplasia with squamous metaplasia (arrows). (c). Endometrial cysts (labelled c) and mucosal bridges traversing the lumen were observed in all the Cdc73+/− mice with neoplasms (b-e). (d) H&E-stained section of a uterine adenofibroma from a Cdc73+/− mouse with irregular polypoid endometrial projections into the lumen and cyst formation. (E) H&E-stained section of a uterine adenomyoma with glandular endometrium and irregular endometrial polyps projecting into the lumen. (f) Increased nuclear Ki-67 expression was observed in the endometrium and myometrium of uteri from Cdc73+/− mice (brown nuclei, arrows) with haematoxylin nuclear counterstain. The interface of endometrium (Endo) and myometrium (Myo) is indicated by a solid red line. (k) Immunostaining for parafibromin in a section of a uterus from a Cdc73+/+ mouse demonstrating normal endometrial expression of parafibromin. (g) Parafibromin-stained sections of a uterine tumour from a Cdc73+/− mouse demonstrating reduced parafibromin expression in the endometrium. (h) Section of a uterus from a Cdc73+/− mouse demonstrating normal expression of progesterone receptor in the endometrium. (i) Loss of endometrial progesterone receptor expression in a uterine tumour from a Cdc73+/− mouse. (j) Immunostaining for the proliferation marker Ki-67 in a section of a uterus from a Cdc73+/− mouse (brown nuclei, arrows) with haematoxylin nuclear counterstain. The interface of endometrium (Endo) and myometrium (Myo) is indicated by a solid red line. (k) Increased nuclear Ki-67 expression was observed in the endometrium and myometrium of uteri from Cdc73+/− mice with tumours (arrows). (l) Quantiﬁcation of Ki-67 labelling index in the endometrium and myometrium of uteri from Cdc73+/− mice (blue bars) and from Cdc73+/− mice with tumours (red bars) demonstrated signiﬁcantly higher proliferation in Cdc73+/− mice (**P < 0.01, ***P < 0.001) compared to Cdc73+/+ mice (total (n = 8) fields of view from four mice per genotype for: Cdc73+/− endometrium n = 84, Cdc73+/− myometrium n = 80, and Cdc73+/− endometrium n = 82). All scale bars represent 100 μm; insets have 400× magnification.

MATERIALS AND METHODS

Mouse studies

The generation of the conventional and conditional Cdc73 knockout mouse models has been previously described.10,16 Conventional Cdc73+/− mice established using the embryonic stem cell line RRE190,19 were maintained on a C57BL/6 background for 10 generations to obtain congenic Cdc73+/−/ mice. Cdc73+/− mice19 were mated with parathyroid-specific Cre-expressing, PTH-Cre mice,24 to generate heterozygous PTH-Cre mice. These Cdc73+/−/PTH-Cre mice were intercrossed to generate three genotypes expressing the Cre-recombinase: Cdc73+/−/PTH-Cre, Cdc73+/−/PTH-Cre, and Cdc73+/−/PTH-Cre. All mice were fed a standard diet (RM1 expanded diet, Special Diet Services Ltd., Witham, UK) and kept in accordance with national welfare guidelines and project license restrictions. Specifically, the animal studies were approved by the University of Oxford Ethical Review Committee and were licensed under the Animal (Scientific Procedures) Act 1986, issued by the United Kingdom Home Office Department (PLL 30/2014), and the Institutional Animal Care and Use Committee of the Van Andel Research Institute.

Cdc73+/− and Cdc73+/− mice underwent a full post mortem at ~7 and >17–21 months of age, together with collection of blood samples for serum analysis and collecting of tissues for histological analysis. Cdc73+/−/ PTH-Cre, Cdc73+/−/PTH-Cre and Cdc73+/−/PTH-Cre mice were studied at ~7–12 months and ~20 months of age. Macroscopic and microscopic examinations for HPT-JT associated tumours was undertaken.

Genotype studies

Genotypes of the Cdc73+/− and Cdc73+/− mice were determined by polymerase chain reaction (PCR) analysis of DNA using PCR primers (f 5′-GTCACAAACACAAAGCCTCAG-3′, r 5′-GTACAAGGTCTCGGATATTATTACC-3′ and G 5′-CTGAAAGGCGAAGATGTAAGC-3′) to yield a wild-type band of 321 bp and a mutant band of 289 bp. Reverse transcriptase-PCR (RT-PCR), using total RNA extracted from Cdc73+/− and

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Cdc73+/− kidneys was performed using either Cdc73-specific primers 3f (5′-GACCGACCGAAAAGATCTAC-3′), 9r (5′-AGGCTTTTGACGACAAATG-3′), and rev (5′-CCCAAGTCTGGCGGCGTATT-3′) to yield a wild-type band of 593 bp, or a mutant band of 500 bp (Figure 1b), as described.69 Genotypes of Cdc73+/−/PTH-Cre, Cdc73+/−/PTH-Cre, and Cdc73−/−/PTH-Cre mice were determined by PCR analysis of DNA using primers to detect the presence of LoxP and Cre-recombinase sites as previously described.19,24

Histology and immunohistochemistry

Tissues were fixed overnight in neutral buffered 4% paraformaldehyde before embedding and sectioning for immunohistochemical analysis. Haematoxylin and eosin staining was performed, using previously described methods.60 Commercially available antibodies were obtained and used according to the manufacturer’s instructions (rabbit anti-parafibromin, ASC171A and rabbit anti-parafibromin IHC-00379 (Bethyl, Montebello, TX, USA), rabbit anti-galectin-3 ab53082 (Abcam, Cambridge, UK), rat anti-Ki-67 M7249 (Dako, Glostrup, Denmark), rabbit anti-cyclin D1 clone SP4 (Thermo, Waltham, MA, USA), and rabbit anti-retinoblastoma sc-7905 (Santa Cruz, Heidelberg, Germany)). Colour reaction was developed using secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) or biotinylated rabbit anti-rat antibody and streptavidin/HRP (DakoCytomation, Glostrup, Denmark) and used according to the manufacturer’s instructions to assess for apoptotic cells. Background was stained red/purple. Proliferation analysis was performed, though, UK), and nuclei were counterstained with haematoxylin, as described.

Western blot analysis

Western blot analysis using total protein extracted from tissues of Cdc73+/− and Cdc73−/− mice was performed (Figure 1c), as previously described.60 The ability of the anti-parafibromin antibody to detect parafibromin was validated using siRNA targeting CDC73 (Dharmacon, Amersham, UK).

Statistical analysis

Normally distributed data were analysed by Student’s t-test or ANOVA followed by Tukey’s multiple comparison post-hoc test. A two-tailed Fisher’s exact test was used for 2x2 contingency tables, and Kaplan–Meyer analysis was performed using a two-tailed Log-rank test.50 P-values < 0.05 were considered statistically significant. Sample sizes are stated in the results section and the figure legends. The sample size for the survival study was selected based on a power of 80% to detect a 5% significance level (two-tailed) using equal numbers per group and a hazard ratio of 0.5; no animals were excluded, randomization was not required and blinding was not performed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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