New mouse models for metabolic bone diseases generated by genome-wide ENU mutagenesis

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Abstract Metabolic bone disorders arise as primary diseases or may be secondary due to a multitude of organ malfunctions. Animal models are required to understand the molecular mechanisms responsible for the imbalances of bone metabolism in disturbed bone mineralization diseases. Here we present the isolation of mutant mouse models for metabolic bone diseases by phenotyping blood parameters that target bone turnover within the large-scale genome-wide Munich ENU Mutagenesis Project. A screening panel of three clinical parameters, also commonly used as biochemical markers in patients with metabolic bone diseases, was chosen. Total alkaline phosphatase activity and total calcium and inorganic phosphate levels in plasma samples of F1 offspring produced from ENU-mutagenized C3HeB/FeJ male mice were measured. Screening of 9,540 mice led to the identification of 257 phenodeviants of which 190 were tested by genetic confirmation crosses. Seventy-one new dominant mutant lines showing alterations of at least one of the biochemical parameters of interest were confirmed. Fifteen mutations among three genes (Phex, Casr, and Alpl) have been identified by positional-candidate gene approaches and one mutation of the Asgr1 gene, which was identified by next-generation sequencing. All new mutant mouse lines are offered as a resource for the scientific community.

I. Rubio-Aliaga and W. Hans contributed equally to this study.

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Introduction

Metabolic bone diseases originate from endocrine dysfunctions as well as from heterogeneous determinants, including age, lifestyle, and environmental influences. Bone turnover is physiologically regulated by hormones, cytokines, and growth factors and is under the control of numerous signaling pathways (Chavassieux et al. 2007). Metabolic diseases may have primary or secondary impact on bone mineralization. For investigating disease development and progression and to understand the underlying mechanisms, mice have been shown to serve successfully as model organisms (e.g., Abe et al. 2007; Kurima et al. 2002; Marklund et al. 2010; McGowan et al. 2008). Random N-ethyl-N-nitrosourea (ENU) mutagenesis is a promising approach to obtain mouse models for inherited human diseases (Hrabé de Angelis and Balling 1998). This has been shown in worldwide ENU mutagenesis programs, including bone metabolism, using dual-energy X-ray absorptiometry (DEXA), X-ray analysis, biochemical markers, or the SHIRPA protocol for the phenotyping of ENU mutagenesis-derived C57H/HeJ, BALB/cCR/LAnn, and B57BL/6 J mice (Barbaric et al. 2008; Smits et al. 2010; Srivastava et al. 2003).

Within the large-scale Munich ENU mutagenesis screen more than 850 mutant mouse lines have been isolated, derived from a large-scale genome-wide screen (Hrabé de Angelis et al. 2000) or from an implemented modifier screen on Dll1lacZ knockout mice (Rubio-Aliaga et al. 2007). Our Dysmorphology Screen is focusing on the isolation of new mouse models for hereditary metabolic bone diseases (Fuchs et al. 2006; Lisse et al. 2008).

In previous studies in mice the reliability of biochemical markers for skeletal disorders, including alkaline phosphatase (ALP), has been shown (Srivastava et al. 2001). Combined ALP, total calcium (Ca), and inorganic phosphate (P'i) measurements in serum or plasma are routinely performed in patients with metabolic bone diseases (Table 1).

Ca and P'i homeostasis is balanced by intestinal absorption, mobilization, or binding in bone and renal excretion. Ca levels directly and indirectly influence intestinal phosphate absorption. Much less is known about the influences on P'i homeostasis (Bergwitz and Jüppner 2010). A key role in maintaining phosphate homeostasis is the reabsorption of phosphate from urine into the renal proximal tubules. A previously identified phosphaturic factor, FGF23 (fibroblast growth factor 23), acts as an endocrine hormone on the regulation of P'i reabsorption in the kidney and on renal vitamin D metabolism (ADHR Consortium 2000; Strom and Jüppner 2008).

Here we describe the results of the Munich ENU Mutagenesis Project to obtain new mutant mouse models for impaired bone metabolism by phenotyping for alterations of at least one of the described plasma parameters as selection criteria.

| Mouse models | +ALP | Ca | P'i | Human disease |
|--------------|------|----|-----|---------------|
| ^Fgf23R176Q  |      | O  | O   | Autosomal dominant hypophosphatemic rickets (ADHR) |
| ^Dmp1tm1Mis |      | O  | O   | Autosomal recessive hypophosphatemic rickets (ARHR) |
| ^Sle34a3tm1Kini |  | O   | O   | Hereditary hypophosphatemic rickets with hypercalciuria (HHR) |
| ^PhexHyptm21 |      | O  | O   | X-linked hypophosphatemic rickets (XLHR) |
| ^Alp1tm1M1a |      | O  | O   | Hypophosphatasia (HPP) |
| ^Col1a1tm1 |      | O  | O   | Paget’s disease of bone (PDB) |
| ^Col1a1tm1 |      | O  | O   | Primary hyperparathyroidism |

Φ unchanged, † increased, ↓ decreased, n no data. ALP alkaline phosphatase, Ca total calcium, P'i total inorganic phosphate

Table 1 Mouse models for metabolic bone diseases with altered ALP activity and Ca and P'i values in plasma and corresponding human diseases with equivalent changes

A Econs and McEnery (1997), ^Lorenz-Depiereux et al. (2006a), ^Lorenz-Depiereux et al. (2010), ^Mejia-Gaviria et al. (2010), ^Lorenz-Depiereux et al. (2006b), ^Delmas (1992), ^Tieder et al. (1985), ^Mornet et al. (2001), ^Whyte (2010), ^Chodoriker et al. (1990), ^Cundy et al. (2007), ^Braga et al. (2004), ^Singer et al. (1998), ^Freeman (1988), ^Goseki-Sone et al. (2005), ^Bilezikian et al. (2005), ^Tiosano and Hochberg (2009)

A Farrow et al. (2011), ^Feng et al. (2008), ^Feng et al. (2003), ^Segawa et al. (2009), ^Lorenz-Depiereux et al. (2004), ^Eicher and Southard (1972), ^Hough et al. (2007), ^Lomeli et al. (2000), ^Narisawa et al. (1997), ^MacGregor et al. (1995), ^Table 4, ^Lisse et al. (2008), ^Chipman et al. (1993), ^Bonadio et al. (1990), ^Liu (1995), ^Badanini et al. (2010), ^Ho et al. (1995)
markers. We isolated 71 new murine models that may be of special value for the development of new therapeutic approaches since a high number of metabolic bone diseases in human patients are caused by point mutations (Marini et al. 2010; Simon-Bouy et al. 2008; Wenkert et al. 2011).

Material and methods

Mice

For this study we used C3HeB/FeJ (C3H) inbred mice purchased originally from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in our animal facility. The mice were housed and handled according to the federal animal welfare guidelines and the state ethics committee approved all animal studies. The mice were kept in a 12/12-h dark–light cycle and provided standard chow ad libitum (TPT total pathogen-free chow #1314: calcium content 0.9 %, phosphate 0.7 %, vitamin D3 600 IE; Altromin, Lage, Germany) and water. Hygienic monitoring was performed following FELASA recommendations (Nicklas et al. 2002). Mutant mouse lines derived from our screen were given internal lab codes and were assigned with official gene symbols and names after the mutation was identified.

ENU mutagenesis

ENU mutagenesis treatment of inbred strain C3H males was as described previously (Aigner et al. 2011). Litters produced from the ENU-treated C3H males (G0) are designated F1 in the following, while offspring produced from confirmed mutant F1 animals are designated G2.

Generation of F1 mice and confirmation of phenotypes in a dominant breeding strategy

The F1 animals investigated for this study were derived from a total of 893 G0 males from 15 different ENU-treated groups. Blood samples of 9,540 F1 animals (4,606 females and 4,934 males) were screened for alterations of total ALP, Ca, and Pi blood plasma levels. F1 mice showing alterations of blood-based parameters were retested after 14 days. Breeding for confirmation of a dominant phenotype was performed as described previously (Aigner et al. 2007).

Blood measurements

Blood samples (250 μl) were obtained from 12-week-old nonfasted anesthetized mice by puncture of the retro-orbital sinus, as already described (Rathkolb et al. 2000). All samples were collected between 9:00 and 11:00 a.m. Plasma analysis of ALP, Ca, and Pi was done using an Olympus AU400 autoanalyzer (Olympus, Hamburg, Germany) and adapted test kits (Klempt et al. 2006). Descriptive data are expressed as mean ± standard deviation. PTH values were analyzed with a Mouse Intact PTH ELISA Kit (TECOmedical, Bünde, Germany).

DXA and X-ray measurement

DXA (pDEXA Sabre, Norland Medical Systems Inc., Basingstoke, Hampshire UK, distributed by Stratec Medizintechnik GmbH, Pforzheim, Germany) and X-ray (Faxitron, Hewlett Packard, Palo Alto, CA, USA) measurements were performed for in-depth analysis in selected mouse lines as described previously (Abe et al. 2006; Fuchs et al. 2011).

Genetic mapping

To map the mutations, ENU-derived mutant mice were outcrossed to wild-type C57BL/6 J (B6) mice, as described previously (Aigner et al. 2009). For linkage analysis, SNP (single-nucleotide polymorphism) genotyping by high-throughput MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) technology supplied by Sequenom (San Diego, CA, USA) was performed with a panel containing 158 markers evenly distributed over the whole genome (Klaften and Hrabé de Angelis 2005). We developed the internal MyGenotype database for statistical SNP data analysis.

Mutation analysis

Castr, Phex, Alpl, and Asgr1 exons were amplified with intronic primers and directly sequenced using BigDye v3.1 cycle sequencing (Applied Biosystems, Life Technologies, Foster City, CA, USA). Casr consists of 7 exons (NM013803), Phex (NM011077) consists of 22 exons, and Alpl (NM007431) consists of 12 exons. All primer sequences are available upon request. The mutation of the BAP005 mutant line was detected by chromosome sorting (CHROMBIOS, Raubling, Germany) and whole-chromosome sequencing on a Genome Analyzer IIx (Illumina, San Diego, CA, USA). DNA extraction from sorted chromosomes 11 was performed overnight at 42 °C with 0.25 M EDTA, 10 % Na lauroyl sarcosine, and 50 μg proteinase K. Extracted DNA was precipitated and resuspended in TE buffer. Paired-end libraries were constructed with the Illumina paired-end DNA sample preparation kit according to the manufacturer’s protocols and as described previously (Eck et al. 2009). Alignment of the reads was performed with the BWA software, and subsequent analysis was performed with the SAMtools package. In total, ~ 82 million reads and ~ 157 million reads were generated for the mutant and control strain, respectively, of which 64 % mapped to the
target chromosome 11 for the mutant strain, while 26 % of the control strain reads were on target. The identified nonsynonymous sequence variation in Asgr1 was confirmed in mutant mice by capillary sequencing.

Statistical analysis

Statistical analysis of parameters of F1 animals and sex- and age-matched wild-type C3H mice were performed using the software package JMP Release 5.1 (SAS Institute, Cary, NC, USA). The reference values were obtained from untreated age-matched C3H wild-type control groups (50 males and 50 females). Single F1 variants for ALP activity and Ca levels were defined by a Z score ≥3 or ≤–3 compared to the age-matched control groups. Mice showing hypophosphatemia were tested three times to confirm Pi changes. A Z score of ≤–2 was taken to select variants for hypophosphatemia. Statistical differences (P values) of the means of ALP, Ca, or Pi blood values between all tested affected mice and nonaffected littersmates of a mutant line were assessed by one-way analysis of variance (ANOVA), t test (giving mean ± SD values), and the Mann-Whitney rank sum test (giving median values) using SigmaStat 3.5 (Systat Software Inc., Chicago, IL, USA).

Results

Overall results and statistics

In order to identify early stages of disturbed bone turnover, we investigated the diagnostic value of routine assays for ALP activity and Ca and Pi levels in the plasma of mice derived from ENU-treated males for its comparability to their use in human patients (Table 1). This table also shows other mouse lines obtained for selected metabolic bone diseases and the observed alterations of plasma parameters in these models. Since we were interested only in mouse lines showing alterations of the bone ALP (bALP) isoform of the measured total ALP enzyme, variants with additional alterations of ALAT (alanine-amino-transferase) and ASAT (aspartate-amino-transferase) levels were excluded from this study.

Two hundred fifty-seven phenodeviants (2.7 %, 87 females and 170 males) of 9,540 F1 animals showed alterations in at least one of the three parameters of interest (ALP, Ca, and Pi) in two repeated blood measurements. One hundred ninety of the 257 (74 %) phenodeviants were mated to wild-type C3H mice in confirmation crosses. In 71 of the mated 190 (37 %) (25 females and 46 males), the observed phenotype was genetically transmitted as a dominant trait (Table 2); however, six of these mutant lines were lost because no mutant male offspring was produced for sperm cryopreservation. For 110 of the mated 190 (58 %) phenodeviants, inheritance could not be confirmed because of sterility (n = 22/110, 20 %), the mice died due to unknown reasons (n = 15/110, 14 %), or the hypothesis of a dominant mutation was excluded (n = 73/110, 66 %). Confirmation crosses for the remaining 9 of the 190 phenodeviants are still underway. Sixty-seven of the 257 (26 %) phenodeviants were not mated due to space limitations; however, their sperm was frozen. Founder F1 mice with a similar phenotype and derived from the identical G0 male were expected to carry the identical mutation. Fifteen mutations have been

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**Table 2** Genetic confirmation crosses and confirmed mutations for F1 variants with alterations of ALP activity and/or Ca and Pi plasma values

| Phenotypea | Confirmation crosses |
|------------|----------------------|
|            | Total number | Confirmed (% of total F1 tested for this phenotype) | Ongoing |
| ALP Ca Pi  |            |                                                      |        |
| † † Ø Ø   | 83          | 28 (33.7)                                             | 1      |
| † † † Ø   | 4           | 2 (50)                                                | 0      |
| † † † †    | 1           | 0                                                     | 0      |
| † † † Ø    | 11          | 1 (100)                                               | 0      |
| † Ø Ø     | 14          | 4 (28.6)                                              | 1      |
| † Ø Ø     | 10          | 6 (60)                                                | 1      |
| † Ø Ø     | 1           | 0                                                     | 0      |
| † Ø Ø     | 20          | 9 (45)                                                | 0      |
| † Ø Ø     | 1           | 0                                                     | 0      |
| † Ø Ø     | 8           | 4 (50)                                                | 2      |
| † Ø Ø     | 3           | 1 (33)                                                | 0      |
| † Ø Ø     | 2           | 1 (50)                                                | 0      |
| † Ø Ø     | 41          | 14 (34.1)                                             | 4      |
| Total     | 190         | 71 (37.4)                                             | 9      |

† high, † low, Ø unchanged, ALP alkaline phosphatase, Ca total calcium, Pi total inorganic phosphate
identified resulting in new alleles of the Phex, Casr, and Alpl genes (Table 3).

New mouse lines carrying mutations of the Phex (phosphate-regulating gene with homologies to endopeptidases on the X-chromosome) gene

Affected animals of the BAP012 (Bone screen Alkaline Phosphatase No. 012) mutant line displayed a significant ($P \leq 0.001$) decrease in plasma $P_i$ levels. Female mutant mice ($n = 42$) exhibited a $P_i$ value of $1.3 \pm 0.2$ mmol/l compared to female wild-type mice ($2.0 \pm 0.3$ mmol/l, $n = 11$). Male mutant mice ($n = 7$) had a $P_i$ value of $1.2 \pm 0.1$ mmol/l compared to $2.0 \pm 0.3$ mmol/l in male wild-type mice ($n = 44$). Mean ALP activity was significantly elevated ($P \leq 0.001$) in female mutants ($266.4 \pm 35.3$ U/l) compared to wild-type littermates ($147.9 \pm 17.9$ U/l), and also in mutant male mice ($370.9 \pm 88.5$ U/l) compared to their wild-type littermates ($120 \pm 8.5$ U/l). In addition to these biochemical alterations, all mutants showed reduced body size, shortened hind limbs, and mild head-tossing behavior as described in other Phex mouse models (Lorenz-Depiereux et al. 2004; Moriyama et al. 2011). Genetic crosses revealed X-linked inheritance of the phenotype. Thus, mutant mice of both sexes were derived from mated mutant females, but from matings of male phenotype. Thus, mutant mice of both sexes were derived from mated mutant females, but from matings of male

calcium-sensing receptor) gene

The BCH002 (Bone screen Calcium High No. 002) line showed a statistically significant increase of Ca levels in mutant animals compared to wild-type littermates ($P \leq 0.001$). Female mutants’ Ca level was $2.9 \pm 0.1$ mmol/l ($n = 23$) compared to $2.43 \pm 0.1$ mmol/l for wild-type littermates ($n = 19$). The male mutant value was $2.87 \pm 0.1$ mmol/l ($n = 20$) compared to the wild-type littermates’ value of $2.41 \pm 0.1$ mmol/l ($n = 20$). Fifty-three percent of female and male mutant BCH002 mice had slightly reduced $P_i$ levels. Histological analysis showed enlarged parathyroid glands in heterozygous mutant mice (Fig. 2a). A group of 11 female (6 mutants, 5 wild types) and 20 male mice (10 mutants, 10 wild types) was tested for PTH values, resulting in significantly raised median PTH values for mutant mice ($P \leq 0.001$): female mutants, $214.9$ pg/ml (25% $203.3$ pg/ml and 75% $265.7$ pg/ml), and wild types, $85.7$ pg/ml (25% $79.6$ pg/ml and 75% $113.1$ pg/ml). Male mutants showed $235$ pg/ml (25% $191$ pg/ml and 75% $409.9$ pg/ml) compared to wild types showing $102.7$ pg/ml (25% $78.8$ pg/ml and 75% $117.2$ pg/ml). So far eight pups were derived from a first heterozygous intercross but no homozygous mutant was found. Mapping analysis of 40 mutant and 20 wild-type BCH002 animals derived from the dominant backcrosses to the B6 strain revealed linkage to chromosome 16 (Table 4), with the highest $\chi^2$ value at the marker rs4186801 (51.47 Mb, mouse genome Build 37.1, UCSC). In this region Casr was the most promising candidate gene for the observed phenotype. DNA sequence analysis of the Casr gene revealed a new heterozygous missense mutation (c.2579T $\rightarrow$ A, p.Ile859Asn) within the protein-coding region of exon 7 (Fig. 2b) of the gene that was not present in wild-type C3H and B6 mice. CASR belongs to the family of G-protein-coupled receptors (GPRCs) and is an integral membrane protein that senses changes in the extracellular calcium concentration to parathyroid cells.

In addition, six new alleles of the Casr gene were isolated in other mouse lines (BCH003, BCH004, BCH007, BCH011, BCH013, and BCH014) (Table 3) creating an allelic series for functional analysis of the gene. Median PTH levels were significantly increased ($P = 0.010$) in first testings of BCH013 female mutants ($n = 6$), showing $175.2$ pg/ml (25% $143.2$ pg/ml and 75% $198.33$ pg/ml) compared to wild types ($n = 4$) showing $50.61$ pg/ml (25% $48.05$ pg/ml and 75% $56.96$ pg/ml). Male mutants ($n = 10$) displayed $100.98$ $\pm$ $30.765$ pg/ml and wild types ($n = 9$) $55.485$ $\pm$ $14.734$ pg/ml. For all other

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| Line name | Variant phenotype | Additional phenotype, comment | Transmission (%) |
|-----------|-------------------|--------------------------------|-----------------|
| BAP001    | High ALP          |                                | <20             |
| BAP002    | High ALP, high Ca |                                | 64              |
| BAP003    | High ALP, high Ca | Mapped on Chr 4 between SNP markers rs28307021 and rs3711383 (101.16–141.90 Mb, mouse genome Build 37.1, UCSC) | 62 |
| BAP004    | High ALP and/or high Ca and/or low Pi | All variants with auricle degeneration when >4 months old; reduced body size; mapped on Chr 4 between SNP markers rs28056583 and rs13469808 (86.81–117.55 Mb, mouse genome Build 37.1, UCSC) | 75 |
| BAP005    | High ALP          | Mutation of the Asgr1 (asialoglycoprotein receptor 1) gene, c.815A > G; p.Tyr272Cys | 92 |
| BAP006    | High ALP          | Identical G0 as BAP007; counted with BAP006 as one line | lost |
| BAP007    | High ALP          | Identical G0 as BPL001 | 71 |
| BAP008    | High ALP          | All variants with auricle degeneration when >4 months old; reduced body size | 32 |
| BAP009    | High ALP          | All variants show circling behavior, reduced body size | lost |
| BAP010    | High ALP          |                                | 32 |
| BAP011    | High ALP          |                                | 100 |
| BAP012    | High ALP, low Pi  | All variants with reduced body size | <20 |
| BAP013    | High ALP          |                                | 27 |
| BAP014    | High ALP          | Significantly more males born and affected; offspring of heterozygous intercrosses with reduced body size, ALP very high; mapped on Chr 9 between SNP markers rs3023207 and rs3673055 (37.50–96.23 Mb, mouse genome Build 37.1, UCSC) | 60 |
| BAP015    | High ALP          |                                | lost |
| BAP016    | High ALP          |                                | 70 |
| BAP017    | High ALP          |                                | <20 |
| BAP018    | High ALP          |                                | 96 |
| BAP019    | High ALP          |                                | 29 |
| BAP020    | Low ALP           | Synonymous sequence variation in exon 10 of the Alpl (alkaline phosphatase, liver/bone/kidney) gene, c.1098A > T; p.Lys50X | 100 |
| BAP021    | High ALP          |                                | 44 |
| BAP022    | High ALP          |                                | 100 |
| BAP023    | Low ALP           | Missense mutation in exon 7 of the Alpl (alkaline phosphatase, liver/bone/kidney) gene, c.755T > G; p.Leu251Pro; Identical G0 animal as BAP021 and BCH009 | 100 |
| BAP024    | High ALP, low Pi  | All variants with reduced body size, circling behavior. Missense mutation in exon 22 of the Phex (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) gene, c.2197T > C; p.Cys733Arg | 100 |
| BAP025    | High ALP          |                                | 100 |
| BAP026    | Low ALP           | Splice site mutation in intron 9 of the Alpl (alkaline phosphatase, liver/bone/kidney) gene, c.997+2T > G | 85 |
| BAP027    | Low ALP           | Missense mutation in exon 10 of the Alpl (alkaline phosphatase, liver/bone/kidney) gene, c.1194T > A; p.Ile395Asn | 100 |
| BAP028    | High ALP          | All mutants with reduced body size | 100 |
| BAP029    | High ALP          |                                | 30 |
| BAP030    | High ALP          | All mutants with reduced body size | 56 |
| BAP031    | High ALP          |                                | 52 |
| BAP032    | Low ALP           | Missense mutation in exon 11 of the Alpl (alkaline phosphatase, liver/bone/kidney) gene, c.1217A > G, p.Asp406Gly | 90 |
| BCH001    | High Ca           |                                | <20 |
| BCH002    | High Ca, low Pi   | Missense mutation in exon 7 of the Casr (calcium-sensing receptor) gene, c.2579T > A; p.Ile859Asn. Some intercrosses derived offspring with reduced body size, gray fur | 100 |
| Line name | Variant phenotype | Additional phenotype, comment | Transmission (%)<sup>a</sup> |
|-----------|-------------------|--------------------------------|-------------------------------|
| BCH003    | High Ca           | Missense mutation in exon 3 of the Casr (calcium-sensing receptor) gene, c.295G > T, p.Asp99Tyr. Identical G0 as BCH006 | 100                          |
| BCH004    | High Ca           | Nonsense mutation in exon 4 of the Casr (calcium-sensing receptor) gene, c. 366G > T, p.Glu456X | 93                           |
| BCH005    | High Ca           | Identical G0 as BCH003; counted with BCH003 as a single line | 22                           |
| BCH006    | High Ca           | Missense mutation in exon 4 of the Casr (calcium-sensing receptor) gene, c.626T > C; p.Val208Ala. Some intercrosses derived offspring show reduced body size. | 100                          |
| BCH008    | High Ca           |                             | 78                           |
| BCH009    | High Ca           | Identical G0 animal as BAP021 and BAP023 | <20                          |
| BCH010    | High Ca           |                             | 20                           |
| BCH011    | High Ca           | Nonsense mutation in exon 7 of the Casr (calcium-sensing receptor) gene, c.2017C > T, p.Gln673X | 100                          |
| BCH012    | High Ca           |                             | <20                          |
| BCH013    | High Ca           | Missense mutation in exon 3 of the Casr (calcium-sensing receptor) gene, c.296A > G, p.Asp99Gly | 100                          |
| BCL001    | Low Ca, high ALP  |                             | 100                          |
| BCL002    | Low Ca            |                             | 50                           |
| BPH001    | High P<sub>i</sub>|                             | lost                         |
| BPL001    | Low P<sub>i</sub> | Identical G0 animal as BAP008 | <20                          |
| BPL002    | Low P<sub>i</sub> | Mapped on Chr 16 between SNP markers rs4186801 and rs4199268 (51.47–69.80 Mb, mouse genome Build 37.1, UCSC) | 38                           |
| BPL003    | Low P<sub>i</sub> |                             | <20                          |
| BPL004    | Low P<sub>i</sub> | Mapped on Chr 3 between SNP markers rs13477178 and rs13477321 (69.55–109.00 Mb, mouse genome Build 37.1, UCSC) | 63                           |
| BPL005    | Low P<sub>i</sub> |                             | 46                           |
| BPL006    | Low P<sub>i</sub> | All mutants with reduced body size; mapped on Chr 14 between SNP markers rs30406796 and rs30865397 (22.92–74.08 Mb, mouse genome Build 37.1, UCSC) | 73                           |
| BPL007    | Low P<sub>i</sub> |                             | 53                           |
| BPL008    | Low P<sub>i</sub> | All mutants with reduced body size; mapped on Chr 8 between SNP markers rs13479952 and rs13479998 (103.43–116.69 Mb, mouse genome Build 37.1, UCSC) | 37                           |
| BPL009    | Low P<sub>i</sub> |                             | 40                           |
| BPL010    | Low P<sub>i</sub> |                             | 100                          |
| BPL011    | Low P<sub>i</sub> |                             | 67                           |
| BPL012    | Low P<sub>i</sub> |                             | 50                           |
| BPL013    | Low P<sub>i</sub> |                             | 50                           |
| BPL014    | Low P<sub>i</sub> |                             | 100                          |
| SAP003    | High ALP          |                             | <20                          |
| SAP004    | Low Ca, low P<sub>i</sub> |                             | <20                          |
| SAP005    | High ALP          |                             | lost                         |
| SAP006    | High ALP          |                             | 41                           |
| SAP007    | Low ALP           | Missense mutation in exon 12 of the Alpl (alkaline phosphatase, liver/bone/kidney) gene, c.1357A > G; p.Thr453Ala | 100                          |
| SAP008    | High ALP          |                             | 62                           |
| SCA001    | High Ca           |                             | 49                           |
| SMA010    | High ALP, high Ca | All variants with reduced body size (Z score < −2) | 31                           |
| TRE002    | High ALP          | All mutants trembling, high ALP probably secondary effect | 100                          |

All mouse lines listed in alphabetical order of the internal lab names

* According to dominant inheritance 50 % mutant offspring corresponds to 100 % transmission of the phenotype
mouse lines with mutations of the \textit{Casr} gene, PTH data are underway. The missense and nonsense mutations of these mouse lines were located in exons 3, 4, 5, and 7 of the \textit{Casr} gene (Table 3). Four sequence variations were located in exons 7, 10, or 12 (BAP020, BAP023, BAP027, SAP007) and one affects the splice site in intron 9 (BAP026).

Other mouse lines and mutations

Female and male mutant mice of the BAP005 line showed statistically significant ($P \leq 0.001$) increased mean ALP activity. The value in female mutants ($n = 25$) was $233 \pm 21$ U/l compared to $136 \pm 14$ U/l in wild-type littermates ($n = 32$), and in male mutants ($n = 39$) ALP activity was $188 \pm 19.81$ U/l compared to $104.5 \pm 10.43$ U/l in their wild-type littermates ($n = 36$). The mouse line breeds homozygous offspring with very high ALP activities. Homozygous females derived from heterozygous intercrosses showed mean ALP activity of $587 \pm 39$ U/l ($n = 12$), and ALP activity in homozygous males was $482 \pm 51$ U/l ($n = 21$). SNP mapping revealed a region between the markers rs26982471 and rs27000576 (53.99–114.33 Mb, mouse genome Build 37.1, UCSC) on chromosome 11. Sorting of chromosome 11 and whole chromosome 11 sequencing on a GAIIx next-generation sequencing machine revealed a new missense mutation in the \textit{Asgr1} (asialoglykoprotein receptor 1) gene within the translated region (c.815A $\rightarrow$ G, p.Tyr272Cys). The mutation was sequenced in 16 BAP005 mutant mice, but neither in 4 wild-type littermates, nor in additional 4 wild-type mice from different inbred strains (BALB/c, DBA/2, FVB, SJL).

For eight additional mouse lines (BAP002, BAP003, BAP004, BAP014, BPL004, BPL006, BPL008, and TRE002) showing high ALP activity, low P$_i$, and high or low Ca values as a phenotype, genetic mapping has been finished (Table 3) and sequencing of candidate genes is in progress. For selected mouse lines we will include exome sequencing to find the causative mutation.

Discussion

In this study we described a large-scale ENU mutagenesis screen (Soewarto et al. 2009), with the main focus on malfunctioning bone turnover. In other projects murine models for disturbed bone metabolism were obtained by gene targeting (Daroszewska et al. 2011; Ducy et al. 1996; Forlino et al. 1999; Kato et al. 2002), transgene insertions (Imanishi et al. 2001; Rauch et al. 2010) or spontaneous mutations (Eicher et al. 1976; Marks and Lane 1976). Here, we isolated 71 new mouse models by screening for

the protein-coding region of the \textit{Alpl} gene on chromosome 4 (c.1217A $\rightarrow$ G, p.Asp406Gly) (Fig. 3b). This mutation was not found in wild-type C3H littermates or in wild-type B6 mice. We isolated five additional mouse lines carrying new alleles of the \textit{Alpl} gene (Table 3). Four sequence variations were located in exons 7, 10, or 12 (BAP020, BAP023, BAP027, SAP007) and one affects the splice site in intron 9 (BAP026).
alterations of total ALP activity and total Ca and Pi values in plasma of 9,540 F1 mice. Our results demonstrate that malfunctions of bone metabolism in mice may be efficiently detected by the analysis of human standard clinical chemical parameters.

In this study the highest fraction of new mouse lines revealed alterations of total ALP activity (Table 3). Since these mouse lines discriminated in phenotype expression and occurrence of additional phenotypes, the phenotypes presumably depend on different molecular mechanisms.
Total ALP was chosen as a parameter of interest since elevated ALP activity is the most frequently measured parameter for human Paget’s disease (Langston and Ralston 2004), X-linked hypophosphatemic rickets (XLHR) (Jonsson et al. 2003; Mäkitie et al. 2003), autosomal dominant hypophosphatemic rickets (ADHR) (Econs and McEnery 1997; Imel et al. 2007; Kruse et al. 2001), and type I osteoporosis (Avbersek-Luznik et al. 2007; Pedrazzoni et al. 1996).

Screening for alterations of total Ca and Pi values without changes of ALP activity resulted in 34 mutant lines with confirmation of the observed phenotype (Table 2). While the Ca parameter was easy to measure, Pi values were artificially elevated after plasma storage for longer than 1 day, freezing of the samples, or hemolysis. Metabolic bone diseases may be reflected in changes of more than one parameter, and very often two or three of the parameters of interest showed alterations in the same individual mouse line, as is commonly observed in human patients (Table 1).

In our screen we obtained new mouse models for hypophosphatemia, hyperparathyroidism, and hypophosphatasia. Despite the large number of existing mouse models for XLHR, there are still open questions on the mechanism of PHEX in renal phosphate wasting, abnormal vitamin D metabolism, and matrix mineralization (Addison et al. 2010; Brownstein et al. 2010). The C3HeB/FeJ-AlplBAP032 and C3HeB/FeJ-PhexBAP012 and C3HeB/FeJ-PhexBAP024 mutant lines represent two new mutant mouse lines with novel point mutations modeling XLHR in addition to previously published models (Carpinelli et al. 2002; Lorenz-Depiereux et al. 2004; Xiong et al. 2008).

![DNA sequence analysis of the Alpl gene exon 11 revealed a new heterozygous missense mutation (c.1217A > G, p.Asp406Gly). Variant is marked by an asterisk.](image)

**Table 4** Statistical analysis of SNP mapping data of the C3HeB/FeJ-CasrBCH002 mutant line obtained by MyGenotype database

| Chromosome (marker) | Highest $\chi^2$ | Highest -log10(P) |
|---------------------|------------------|------------------|
| 1 (rs31593281)      | 5.57             | 1.74             |
| 2 (rs3691120)       | 1.80             | 0.75             |
| 3 (rs3685081)       | 1.72             | 0.72             |
| 4 (rs28307021)      | 0.78             | 0.42             |
| 5 (rs32067291)      | 3.60             | 1.24             |
| 6 (rs13478606)      | 3.76             | 1.28             |
| 7 (rs13479476)      | 1.09             | 0.53             |
| 8 (rs13479998)      | 8.02             | 2.34             |
| 9 (rs3023207)       | 5.00             | 1.60             |
| 10 (rs13480484)     | 4.45             | 1.46             |
| 11 (rs27000576)     | 9.38             | 2.66             |
| 12 (rs6194112)      | 7.36             | 2.18             |
| 13 (rs29566800)     | 4.45             | 1.46             |
| 14 (rs30482696)     | 2.17             | 0.85             |
| 15 (rs13482484)     | 9.38             | 2.66             |
| 16 (rs4186801)      | 28.17            | 6.96             |
| 17 (rs13483097)     | 4.79             | 1.54             |
| 18 (rs13483484)     | 0.82             | 0.44             |
| 19 (rs6339594)      | 2.69             | 1.00             |

![ALP blood activities (mean ± SD U/l) in female mutant (N = 8), female wild-type (N = 9), male mutant (N = 12), and male wild-type (N = 10) mice. Mean ± SD ALP activities were: female mutants 47 ± 5.8 U/l (∗∗∗ < 0.001); female wild-types 157.8 ± 7.9 U/l; male mutants 38.4 ± 6.3 U/l (∗∗ < 0.001); male wild-types 129.5 ± 10.1 U/l (t-test).](image)
(Pollak et al. 1994). Approximately two thirds of FHH patients showed loss-of-function mutations involving the 3,234-bp coding region of the CASR gene (D’Souza-Li et al. 2002). Individuals with HP and FHH discriminate in creatinine clearance and serum magnesium values, both being higher in FHH (Marx et al. 1981). It has been demonstrated that individuals with FHH are heterozygous, and children within these families with severe neonatal primary hyperparathyroidism (NSHPT) are homozygous for CASR mutations (Janicic et al. 1995; Pollak et al. 1993). Mice with tissue-specific deletion of Casr in the parathyroid gland and bone exhibited profound bone defects (Chang et al. 2008). C3H;102CasrNaf/H mice carry an activating ENU-derived Casr point mutation that exhibits hypocalcemia, hyperphosphatemia, cataracts, and ectopic calcifications (Hough et al. 2004). We obtained the first presumed loss-of-function point mutation isolated in C3HeB/FeJ-CasrBCH002 mice that is supposed to model human FHH. Since the mouse line has been bred more than 15 generations and because we found six other independent Casr mutations for this phenotype, it is more likely that the consistent phenotype is due to the isolated point mutation of the Casr gene. Heterozygous C3HeB/FeJ-CasrBCH002 mice exhibited high Ca and PTH values similar to targeted Black Swiss/129SvJ Casavr–/– and Casr–/– mice (Ho et al. 1995), but, in addition, they showed enlarged parathyroid glands described only for Casr–/– mice. Further heterozygous intercrosses are required to find out if homozygous C3HeB/FeJ-CasrBCH002 mice are viable, which is not the case in Casr–/– mice. This would raise the opportunity to obtain a mouse model for NSHPT. More than 270 mutations have been described so far in the human CASR mutation database (www.casrdb.mcgill.ca; Nakajima et al. 2009), and interestingly most of the human mutations were found in exons 4 and 7. The mouse lines carrying Casr mutations obtained in our screen showed slight differences in the expression of the phenotype. Additional studies on phenotypical and histological traits will help to discriminate between the different effects of each point mutation on the severity of hyperparathyroidism and concomitantly to improve our understanding of CASR mutations in human patients.

Heterozygous C3HeB/FeJ-AlplBAP032 mice showed a statistically significant reduction of ALP activity in plasma without additional phenotypes, as observed in heterozygous Akp2Hpp+/– mice derived in an ENU mutagenesis screen on C3H/HeH background (Hough et al. 2007). In Akp2Hpp+/– mice, an Alpl loss-of-function mutation led to the rare disease hypophosphatasia (HPP) which displays reduction of plasma ALP activities to about 50 % in Akp2Hpp+/– and a stronger reduction in Akp2Hpp+/–/– mice. Akp2Hpp+/– mice were radiographically and histologically indistinguishable from wild-type mice at different time points, as were 16-week-old C3HeB/FeJ-AlplBAP032 mice in DEXA and X-ray analysis. Interestingly, we observed a stronger ALP reduction in heterozygous C3HeB/FeJ-AlplBAP032 mice than in Akp2Hpp+/– mice, with ALP activities in female and male mutant mice reduced to 29 % of that found in wild-type littermates. Severe HPP forms are characterized by hypominerlization, rickets, seizures, and nephrocalcinosis due to hypercalcubria (Beck et al. 2009). Alpl–/– mice showed a reduction in body size, no detectable ALP levels, and lethality prior to weaning, whereas Alpl–/+ mice appeared healthy (Narisawa et al. 1997). The identical point mutation of C3HeB/FeJ-AlplBAP032 mice has also been described for a patient with HPP (Tailandier et al. 2000). Heterozygous C3HeB/FeJ-AlplBAP032 mice presumably model mild adult HPP. The mouse line was bred for more than ten generations, showing full penetrance of the phenotype in all litters. A multitude of diverse point mutations, deletions, and insertions of the human TNSALP gene causing HPP are listed in the hypophosphatasia database (www.sesep.uvsq.fr/03_hypo_mutations.php). The diversity of published human point mutations emphasizes the importance of mouse models for further investigations on physiological functions and cellular mechanisms of Alpl regions involved in collagen and Ca binding. Interestingly, we isolated in addition one silent mutation in the BAP020 mouse line (Table 4) showing the expected phenotype. No additional Alpl mutations were found in this mouse line. Alpl mRNA and translation of ALP were not analyzed so far.

Since only total ALP can be tested in mice so far, we probably will isolate mouse lines showing alterations other than the bone ALP isoform. High alterations of plasma ALP activities without any additional phenotypes, as observed in homozygous animals of the C3HeB/FeJ-Asgr1BAP005 line, have not been published in mice before. It has been described in patients with chronic liver disease that the adult intestinal ALP isoenzyme was increased due to the reduced efficiency or numbers of asialoglycoprotein receptors (Moss 1994). Thus, the mutation of the gene in BAP005 mice seems to cause alterations of the intestinal ALP isoform as a secondary effect. ASRG1 mutations may be responsible for high ALP activities of so far unknown reasons in humans without any skeletal disorders (Panteghini 1991) or may cause benign familial hyperphosphatasemia (Siraganian et al. 1989).

We have to consider bone as an active metabolic organ with a possible influence on metabolism in diseases of disturbed bone turnover (Ferron et al. 2010; Fulzele et al. 2010). For this reason, systematic analysis of all organ systems, as in the German Mouse Clinic (Gailus-Durner et al. 2005), might provide new insights into the actions in these pathways. Our mouse models will be archived by the European Mouse Mutant Archive (EMMA) and are available (www.emmanet.org) for the scientific community.
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