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Immortalization and characterization of osteoblast cell lines generated from wild-type and *Nmp4*-null mouse bone marrow stromal cells using murine telomerase reverse transcriptase (mTERT)

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Abstract

Intermittent parathyroid hormone (PTH) adds new bone to the osteoporotic skeleton; the transcription factor *Nmp4*/CIZ represses PTH-induced bone formation in mice and as a consequence is a potential drug target for improving hormone clinical efficacy. To explore the impact of *Nmp4*/CIZ on osteoblast phenotype, we immortalized bone marrow stromal cells from wildtype (WT) and *Nmp4*-knockout (KO) mice using murine telomerase reverse transcriptase. Clonal lines were initially chosen based on their positive staining for alkaline phosphatase and capacity for mineralization. Disabling *Nmp4*/CIZ had no gross impact on osteoblast phenotype development. WT and KO clones exhibited identical sustained growth, reduced population doubling times, extended maintenance of the mature osteoblast phenotype, and competency for differentiating toward the osteoblast and adipocyte lineages. Additional screening of the immortalized cells for PTH-responsiveness permitted further studies with single WT and KO clones. We recently demonstrated that PTH-induced *c-fos* femoral mRNA expression is enhanced in *Nmp4*-KO mice and in the present study we observed that hormone stimulated either an equivalent or modestly enhanced increase in *c-fos* mRNA expression in both primary null and KO clone cells depending on PTH concentration. The null primary osteoblasts and KO clone cells exhibited a transiently enhanced response to bone morphogenetic protein 2 (BMP2). The clones exhibited lower and higher expressions of the PTH receptor (*Pthr1*) and the BMP2 receptor (*Bmpr1a*, *ALK3*), respectively, as compared to primary cells. These immortalized cell lines will provide a valuable tool for disentangling the complex functional roles underlying *Nmp4*/CIZ regulation of bone anabolism.

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INTRODUCTION

The response of the osteoblast to parathyroid hormone (PTH) is a necessary step in stimulating the normal process of bone remodeling and, in the case of osteoporosis therapy, bone restoration, but the molecular mechanisms underlying the response of the osteoblast to intermittent PTH are not fully understood (de Paula and Rosen, 2010; Datta and Abou-Samra, 2009; Wang et al., 2007). Several recent studies suggest that exogenously administered intermittent PTH may, in part, enhance the response of osteoblasts to locally produced bone morphogenetic protein 2 (BMP2), which in turn enhances osteogenesis (Kempen et al., 2010; Nakao et al., 2009; Horiuchi et al., 2004).

The nucleocytoplasmic shuttling transcription factor nuclear matrix protein 4/cas interacting zinc finger protein (Nmp4/CIZ, hereafter referred to as Nmp4 for simplicity) represses PTH- and bone morphogenetic protein 2 (BMP2)-induced bone formation in mice in part by suppressing osteoblast activity (Childress et al., 2011; Robling et al., 2009; Morinobu et al., 2005). Female wild-type (WT) and *Nmp4*-knockout (KO) mice treated with recombinant human PTH (1–34) (30 ug/kg/day s.c.) for 7 weeks (10–17 wks of age) exhibited significantly increased whole body, femur, tibia and spine BMD and BMC, however, null mice exhibited a significantly greater gain in femoral, tibial, and vertebral trabecular bone than the WT mice (Childress et al., 2011; Robling et al., 2009). This increase in hormone-stimulated bone formation was accompanied by enhanced serum osteocalcin levels, a biochemical marker for osteoblast activity, consistent with in vitro data showing that primary femur-derived osteoblasts lacking Nmp4 express higher levels of alkaline phosphatase and form more mineralized nodules in culture than cells derived from WT animals (Morinobu et al., 2005). Additionally, BMP2-induced bone formation on adult mouse calvariae in vivo was enhanced in the *Nmp4*-null mice (Morinobu et al., 2005).

Nmp4 regulation of bone anabolism is complex (Childress et al., 2010). We have previously shown that introducing mutations into the Nmp4 cis-element within the PTH response region of the rat MMP13 promoter attenuates basal activity of a promoter-reporter construct in UMR-106 rat osteoblast-like cells while over-expression of Nmp4 in these cells enhances the basal activity of the WT promoter-reporter (Shah et al., 2004). Paradoxically, this same mutation enhances the *MMP13* promoter-reporter response to PTH as well as PGE₂ and over-expression of this transcription factor represses hormone-induced activity (Shah et al., 2004). Similarly, *Nmp4*-KO mice show an enhanced PTH-induced increase in c-fos and Fra-2 RNA suggesting that Nmp4 is also damping the activation of these genes during osteoblast response to hormone (Childress et al., 2011). However, whether these changes represent an alteration in osteoblast response and/or other cell types remains to be determined.

To disentangle the cellular basis of Nmp4 repression of PTH-induced bone formation we immortalized specific lineages of the marrow from both WT and null mice. In the present study we established clonal telomerase-immortalized mouse bone marrow stromal cell lines (BMSC) from both genotypes. Ectopic expression of telomerase in BMSCs maintains the phenotype and extends the life span of the cell providing a convenient and cost-effective preparation for cellular and molecular studies (Huang et al., 2008; Abdallah et al., 2005; Simonsen et al. 2002). We chose three WT and three KO clones, from 30 separate lines based on their expression of alkaline phosphatase and their capacity to mineralize. We

evaluated these six lines for their growth, population doubling times, extended maintenance of the mature osteoblast phenotype, and competency for differentiating toward the osteoblast and adipocyte lineages. From these six lines we chose a single WT clonal line and a single KO clonal line based on their comparative PTH-responsiveness to primary cells and used these clones for further studies with PTH and BMP2.

MATERIALS AND METHODS

Cell culture and reagents

Primary bone marrow stromal cell (BMSC) cultures were prepared from femurs of 6 to 8 wk-old male wild type (WT) C57BL/6J mice obtained from the Jackson Laboratory, Bar Harbor, ME and from *Nmp4*-knockout (KO) mice (Robling et al., 2009). The excised bones were briefly rinsed in 70% EtOH and placed in Hanks Balanced Saline Solution (Mediatech Inc, Manassas, VA) at room temperature. The muscle and connective tissue were detached, and the distal and proximal ends of the femurs removed. The marrow was flushed with α -MEM media (GibcoBRL, Grand Island, NY) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 25 mg/ml amphotericin, 2 mM L-glutamine (Gibco BRL), and 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO). These cells were plated at 3,000–6,000 cells/mm², passage zero (P₀). Five days after initial seeding the cells were supplied with fresh media, which contained ascorbic acid (50 μ g/ml) if the osteogenic phenotype was to be evaluated. The clonal osteoblast-like cell line MC3T3-E1 (clone 4, CRL-2593, from the American Tissue Culture Collection [ATCC]) was maintained in α -MEM supplemented as described above, including ascorbic acid. NIH3T3 cells (murine embryonic fibroblasts, ATCC, CRL-1658) were maintained in Dulbecco's Modified Eagle Medium, high glucose (Invitrogen, Carlsbad, CA), supplemented with 3.7 g/L NaHCO₃, 10% calf serum (Sigma-Aldrich, St. Louis, MO), 100 IU/ml penicillin, 100 μ g/ml streptomycin, 25 μ g/ml amphotercin, and 2mM L-Glutamine (Hyclone Laboratories, Inc, Logan UT). The packaging cell line ecotropic Ψ NX Eco was cultured in Dulbecco's Modified Eagle Medium, high glucose (Invitrogen), supplemented with 3.7 g/L NaHCO₃, 10% fetal calf serum, 1 mM sodium pyruvate, 2mM L-glutamine, 100 μ g/ml streptomycin, and 100 IU/ml penicillin (Mediatech, Inc. Herndon, VA). All cell cultures were maintained in humidified 95% air/5% CO₂ at 37°C and the media changed three times weekly. Protocols for obtaining bone cells from the experimental animals were approved by Institutional Animal Care and Use Committees (IACUC) of the Indiana University Schools of Medicine and Dentistry.

Immortalization of BMSCs using (mTERT)

The catalytic subunit of murine telomerase reverse transcriptase (mTert) cDNA was excised from the EcoRI site of pCR-BluntII-TOPOTM (Open Biosystems, Huntsville, AL) and inserted into the EcoRI site of the retroviral vector pBabe-puro (Addgene Inc Cambridge, MA, plasmid #1764). Sequence fidelity of mTert-pBabe-puro was confirmed by automated DNA sequencing (Biochemistry Biotechnology Facility, Indiana University School of Medicine) using mTert 1, mTert 2, 5' and 3' pBabe primers (Table 1). The mTert-pBabe-puro plasmid was transfected into 70% confluent ecotropic Ψ NX Eco packaging cells (a kind gift from DW Clapp, Cancer Research Institute, Indianapolis, IN), using calcium phosphate precipitation (ProFection® Mammalian Transfection System-Calcium Phosphate, Promega, Madison, WI). After 48 hrs, the viral supernatants were harvested and filtered (0.45 μ m). The mTert-pBabe-puro viral titers were determined using NIH3T3 cells with varied dilutions of retroviral supernatants.

BMSCs (P₀), derived from a single WT male mouse and a single null male animal were seeded at 6000 cells/mm² into 100 $\times$ 20 mm culture dishes and maintained in complete α -MEM medium without ascorbic acid for 10–14 days. The cells at 70–80% confluence were

exposed to viral supernatant (MOI, multiplicity of infection of 8) for 6 hrs in the presence of 4 µg/ml polybrene, after which the medium was replaced. After incubation for 24 hours, fresh virus suspensions were added for an additional 6 hrs with a subsequent change of the medium. Cells were placed under puromycin selection (1 µg/ml) 48 hrs after the final transduction and maintained under this selection agent for 2–3 weeks. The surviving cells were isolated using cloning cylinders and the populations expanded; 12–15 distinct clonal lines were generated for both the WT and *Nmp4*-KO genotypes. These clones were characterized after 8 serial passages. Three WT clones (*Nmp4*^{WT} C10, C11, C13) and three *Nmp4*-KO clones (*Nmp4*^{KO} C1, C4, C7) were selected for further study based on preliminary characterization of their capacity for growth and mineralization.

Analysis of genotype and mRNA expression

To confirm the genotype of our cells, genomic DNA was harvested from primary and clonal cells that had been maintained in culture for 10–14 days using the REDExtract-N-AmpTM Tissue PCR Kit (Sigma) following the manufacturer's protocol. The PCR amplification of *Nmp4* and *neomycin* (*Neo*) genes were used to distinguish WT and null cells; the gene *Rapsn*, receptor-associated protein of the synapse, was used as an internal control to normalize gene amplification (Robling et al., 2009; see Table 1 for primer sequences, product sizes, and PCR reaction conditions).

The osteoblast phenotype of the primary bone cells and the clonal lines were evaluated using RT-PCR; total RNA was harvested from cells maintained under ascorbic acid for 10–14 days using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was converted into cDNA using ProtoScriptTM M-MuLV *Taq* RT-PCR Kit (New England Biolabs® Inc., Ipswich, MA). The specific primer sequences, product sizes, and RT-PCR reaction conditions are listed in Table 1.

Quantitative real-time PCR (qRT-PCR) was used to evaluate PTH- and BMP2-induced changes in gene expression. Total RNA was harvested as described above and converted to cDNA using the First Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK). Real-time PCR primers and probes for *c-fos* (Mm01266652_m1), alkaline phosphatase (Mm01187117_m1), PTHR1 (Mm00441046_m1), BMPR-IA (ALK3, Mm00477650_m1), and the normalizer Rplp2 (Mm03059047_gH) or Rplp0 (Mm00725448_s1) were obtained from Assays-on-DemandTM (Applied Biosystem, Foster City CA). Details of the reaction conditions and the use of the $\Delta\Delta CT$ method for the analysis of the data have been described (Philip et al., 2010; Robling et al., 2009).

Estimation of population doubling time

To evaluate the population doubling times of the primary and immortalized cells, the WT and *Nmp4*-KO primary cells (P_0) were reseeded (P_1) at 5×10^3 cell/well in 24-well plates. The clones were seeded in 6-well plates at 2×10^3 cells/well. Cells were trypsinized and counted at 2, 4, 6, 8, 10 days using a hemocytometer. The cells were evaluated for average population doubling time at day 8, when cultures were 90% confluent. The average population doubling time (T_d) was calculated as $T_d = (T_2 - T_1) \times [\log 2 / \log (q_2/q_1)]$ where $T_2 - T_1$ is the time in hours between measurement of cell counts q_2 and q_1 (Bryant et al., 1958).

Evaluation of osteogenic and adipogenic phenotype of cell lines

In order to evaluate the potential for the osteogenic phenotype cells were maintained in medium supplemented with ascorbic acid (50 µg/ml) for 9 (primary cells) to 14 days (clones) after which cells were stained for alkaline phosphatase activity using naphthol AS-MX phosphate alkaline solution and fast red violet B salt (Leukocyte Alkaline Phosphatase Kit,

Sigma) following the manufacturer's instructions. To assess the capacity for mineralization, cells were maintained in culture under 10 mM beta-glycerophosphate and 50 µg/ml ascorbic acid for up to 21 days post-seeding followed by fixation with 10% buffered formalin and application of von Kossa stain (Brudner et al., 1997). Finally, to evaluate the adipogenic potential of the immortalized clones, cells were cultured in α -MEM medium supplemented as described above; upon reaching 70–100% confluence they were treated with insulin (5 µg/ml), dexamethasone (0.1 µM) and indomethacin (50 µM) for approximately 9–14 days. Cells were fixed with 10% buffered formalin, rinsed with PBS, and stained with Oil Red O to visualize intracellular lipid droplets.

Evaluation of PTH & BMP2 responsiveness

To assess the PTH-induced c-fos response of the WT and *Nmp4*-KO primary osteoblasts and clonal lines, cells were cultured with ascorbic acid for 7–14 days and then treated for 30 minutes with 10nM or 25nM PTH or vehicle control (0.2% BSA/0.1% 1.0 M HCl in saline) in conditioned media. Cells were then processed for qRT-PCR as described above.

To evaluate the impact of *Nmp4* on BMP2-induced alkaline phosphatase expression, WT and *Nmp4*-null clones were seeded into 6-well plates at an initial density of 32 cells/mm² in complete α -MEM without the addition of ascorbic acid. When the cells reached confluence, typically in 2–3 days, treatment with BMP2 (200ng/ml) or vehicle control (4mM HCl, 0.1% BSA) commenced and media containing BMP2 or vehicle was replenished every 3–4 days. The cells were harvested for RNA on either Days 5–6 or Days 12–13 post-seeding. Primary bone marrow cells were seeded at an initial density of 6000 cells/mm² in complete α -MEM without ascorbic acid. Media was changed at Day 5 post-seeding containing either BMP2 (200ng/ml) or vehicle control, and as with the clones media was replenished every 3–4 days. The cells were harvested for RNA on Day 8 or Day 15 post-seeding.

Statistical analysis

To examine in contrast the growth rates of primary osteoblasts and our WT and KO clonal lines we compared slopes of log-transformed cell counts regressed onto the hours from initial seeding using analysis of covariance (ANCOVA), with Tukey's HSD post-hoc test for all pair-wise comparisons, and Dunnett's post-hoc comparison for contrast with nontransformed cells. To compare the magnitude of PTH or BMP2 response between WT and KO cells the average fold induction over vehicle-treated cells were contrasted between the genotypes using a t-test.

RESULTS

The mTert immortalized WT and *Nmp4*-KO bone marrow stromal-derived clones exhibited sustained growth while maintaining an osteoblast phenotype

BMSCs from femurs of *Nmp4*-KO and WT C57BL/6J mice were successfully transduced with the replication-incompetent retrovirus mTert-pBabe-puro (Figure 1A) and selected with puromycin as described in Materials and Methods. We chose three serially passaged WT and three KO clones that were indistinguishable from P₀ primary bone cells based on a preliminary screen evaluating alkaline phosphatase expression and the capacity for mineralization (Figure 1B). As expected the *Nmp4* gene was absent in the KO primary osteoblasts and in the KO clones derived from the null mouse but was present in the WT primary osteoblasts and in the clones established from the WT mouse (Figure 2A). Additionally, no significant *Nmp4* mRNA expression was observed in the null cells using qRT-PCR analysis (data not shown). The expression of *mTERT* mRNA did not decline in the clones after several passages whereas its expression clearly decreased in the primary cells after only four passages (Figures 2B & 2C).

The WT and *Nmp4*-KO clones maintained with ascorbic acid exhibited an osteoblast phenotype over several serial passages as ascertained by comparison of the RNA expression profiles of *Runx2*, *osterix*, *osteocalcin*, *Col1a1*, and *OPN* to the profiles of P₀ primary WT and KO osteoblasts and to MC3T3-E1, an established osteoblast clonal line (Figure 3). In contrast the primary cells exhibited an attenuated osteoblast phenotype after only four passages as indicated by the weakened mRNA expression of the bone-specific transcription factor *osterix* and the osteoblast secreted protein *osteocalcin* (Figure 4A), whereas the expression of these genes in the clones did not appreciably decrease during several serial passages (Figures 4B–4C).

The proliferation capacity of the WT and KO clonal lines was significantly greater than that of the primary osteoblasts (Figures 5A & 5B). The slopes of the log-transformed cell counts regressed onto the hours from initial seeding were significantly steeper for the clonal cells than for the primary cells. There was no difference in growth rate between any of the WT and KO clonal lines. Consistent with this observation we observed no difference in growth rate between the primary WT and KO osteoblast cultures. Finally, the population doubling times of the clonal lines between passages 22–30 were on average 2.7-fold shorter than the doubling time of the primary osteoblasts (Figures 5A & 5B).

The mTert immortalized WT and *Nmp4*-KO bone marrow stromal-derived clones maintained the capacity for differentiating into adipocytes

Primary BMSCs typically exhibit a plasticity in differentiation, particularly between osteoblasts and adipocytes (Delany et al., 2003; Bianco et al., 1999; Lecka-Czernik et al., 1999). Our objective was to immortalize the BMSCs before they committed to any particular lineage, therefore we induced our clones with an adipogenic cocktail of insulin, dexamethasone, and indomethacin as described in Materials and Methods to assess their capacity for exhibiting the adipocyte phenotype. All the WT and KO clones showed significant responsiveness to the cocktail and 50–70% of the cells developed lipid droplets, which is what we typically see with primary WT cultures (Figure 6).

The *Nmp4*-KO primary osteoblasts and clonal null osteoblasts exhibit a modest but significant enhanced response to PTH and BMP2 under specific culture conditions

We have recently reported that *Nmp4*-KO mice exhibit a transiently enhanced PTH-induced increase in femoral *c-fos* RNA 1hr after the last hormone injection subsequent to a daily 2wk treatment regimen compared to WT mice (Childress et al., 2011); thus to determine whether null osteoblasts contribute to this in vivo response profile we challenged primary BMSC-derived WT and null osteoblasts to 10nM hPTH(1–34) or vehicle control for 30min as described in the Materials and Methods. Both the WT and *Nmp4*-KO primary osteoblasts exhibited an ~3–7-fold increase in *c-fos* mRNA expression with no consistent difference between the genotypes (data not shown). We screened our six WT and KO clones for a similar *c-fos* response profile to 10nM PTH and selected the clones *Nmp4*^{WT}11 and *Nmp4*^{KO}7 for further study. We then performed 6 separate experiments: 3 experiments using the primary cells of bone marrow-derived WT and KO osteoblasts and 3 experiments using the *Nmp4*^{WT}11 and *Nmp4*^{KO}7 clonal lines. Cells were treated with 25nM PTH (rather than the 10nM PTH described above) or vehicle control for 30min. Interestingly, we observed that both the primary null osteoblasts and clonal *Nmp4*^{KO}7 exhibited a modest but significantly enhanced response to hormone compared to their WT counterparts in two of three separate trials (Figure 7A and 7B). There was no consistent difference in PTHR1 expression between WT and *Nmp4*-KO cells in either the primary or clonal preparations, however the levels of PTHR1 expression were typically 150–300-fold higher in the primary cells than in clones *Nmp4*^{WT}11 and *Nmp4*^{KO}7 (data not shown).

BMP2-induced bone formation on adult mouse calvariae in vivo was enhanced in the *Nmp4*-null mice and overexpression of *Nmp4* in the osteoblast-like cells MC3T3-E1 suppressed alkaline phosphatase expression (Morinobu et al., 2005; Shen et al., 2002); therefore we evaluated BMP2-induced alkaline phosphatase response in primary bone marrow-derived WT and *Nmp4*-KO osteoblasts and the *Nmp4*^{WT}11 and *Nmp4*^{KO}7 clonal lines. Consistent with the previous study showing that over-expression of *Nmp4* suppressed BMP2-induced alkaline phosphatase expression (Shen et al., 2002) our KO primary osteoblasts and null clonal cells exhibited an enhanced response to this growth factor (Figures 8A & 8B) compared to cells from WT animals. Interestingly, both the WT and KO clones were significantly more responsive to BMP2 than the primary cells and this may be due to the fact that both our WT and KO clones exhibited on average a 2.5–3-fold enhanced expression of the type I BMP receptor ALK3 (Figure 8C). Finally, this enhanced sensitivity of both the KO primary osteoblasts and null clones to BMP2 as compared to the WT cells was dependent on the number of days the cells were in culture. The enhanced response to BMP2 in the primary KO cells was observed after 3 days exposure to growth factor at Day 8 post-seeding; however, WT and KO primary cells exhibited equal increases in alkaline phosphatase expression when evaluated after 10 days exposure to BMP2 and 15 days in culture (data not shown). Conversely, the clonal line *Nmp4*^{KO}7, when compared to the *Nmp4*^{WT}11 line, did not exhibit an enhanced response to BMP2 after 3 days of challenge at Days 5–6 post-seeding (data not shown) but instead showed the exaggerated response to BMP2 after 10 days exposure to growth factor at Day 12–13 post-seeding. Thus *primary* KO cells had a higher response than WT cells after 3 days of exposure, and *clonal* KO cells had a higher response than WT cells after 10 days exposure to BMP2.

DISCUSSION

Nmp4 appears to be a master antagonist to bone anabolism as evidenced by the mildly enhanced skeletal phenotype of the global KO mice (Robling et al., 2009; Morinobu et al., 2005), its suppression of PTH-induced improvement of trabecular architecture (Childress et al., 2011; Robling et al., 2009), its repression of BMP2-mediated ectopic bone formation (Morinobu et al., 2005), and its interference with oscillatory shear stress-induced nuclear translocation of beta-catenin in osteoblasts, a key step in mediating load-induced bone formation (Yang et al., 2010). However, it blocks bone growth by a variety of different cellular and molecular mechanisms. *Nmp4* binds to the minor groove of AT-rich DNA and bends it in the manner of an architectural transcription factor (Alvarez et al., 1998); perhaps this capacity for altering DNA architecture underlies its observed support of the basal expression of both *Mmp13* and *Runx2* in osteoblast-like cells as well as its suppression of the PTH- and BMP2-mediated induction of these genes, respectively (Shah et al., 2004; Shen et al., 2002). The *Nmp4* repression of the shear stress-induced beta-catenin nuclear translocation in osteoblasts appears to be mediated, in part, by regulating the phosphorylation of AKT and GSK-3 β (Yang et al., 2010), which are molecules that regulate the cytoplasmic stability of beta-catenin (Angers & Moon, 2009). *Nmp4* regulation of this response is unlikely a transcriptional effect but perhaps *Nmp4* dampens FAK/PI3K/AKT activation via its interaction with the cytoplasmic adaptor protein p130Cas (Nakamoto et al., 2000). P130cas is a mechanotransducer responsive to changes in focal adhesion activity and a binding partner of FAK (Swada et al., 2006; Birge et al., 2009).

Adding to the complexity of the *Nmp4*-KO phenotype is the recent observation that this protein regulates osteoclastogenesis and mature osteoclast activity (Childress et al., 2011). The bone marrow of null mice contain on average 2-fold more osteoclast progenitors than WT mice and the mature *Nmp4*-KO osteoclasts exhibit an enhanced activity for resorbing dentin when compare with their WT counterparts (Childress et al., 2011).

To initiate studies on resolving the contribution of osteoblasts to the *Nmp4*-KO phenotype from other cells in the marrow microenvironment, we immortalized BMSCs from WT and *Nmp4*-KO mice using mTERT. Our clones were isolated from cells that had not been maintained under ascorbic acid or any other differentiating medium in order to maintain the characteristic phenotypic plasticity of BMSCs (Mödder & Khosla, 2008). Indeed these cells exhibited a significantly enhanced proliferative life-span, compared to primary cells while maintaining the osteogenic and adipogenic potential typical of marrow preparations. When cultured in the presence of ascorbic acid the clones maintained an osteoblast phenotype over several serial passages in contrast to primary cells, which lost the osteoblast phenotype after just a few passages in culture. The present study and previous investigations (Robling et al., 2009; Morinobu et al., 2005) demonstrate that disabling *Nmp4* does not grossly impact the development of the osteoblast phenotype, but instead appears to have a significant effect on its response to anabolic signals.

Experiments with both the primary cells and the clonal lines suggest that the enhanced PTH-induced increase in c-fos femoral mRNA expression previously observed in the *Nmp4*-KO mice (Childress et al., 2011) is in part but not wholly attributable to the response of the osteoblast. The potential importance of the physiologic context of multiple cell types contributing to the gene expression profile of bone may be reflected in the finding that after 2wks of intermittent PTH treatment, hormone induced an 8–12-fold increase in c-fos femoral mRNA 1hr after the last challenge (Childress et al., 2011) compared to the PTH-stimulated 2–3.5-fold increase in naïve primary and clonal cells, 30min after challenge. Additionally, the enhanced hormone-stimulated increase in c-fos expression observed in the null primary and clonal osteoblasts in vitro was sensitive to the concentration of hormone and was observed at 25nM but not 10nM PTH. This was unexpected since disabling *Nmp4* in vivo appears to lower the threshold response to hormone (Robling et al., 2009; Childress et al., 2011). Perhaps osteoblast-osteoclast co-cultures are required to recapitulate the full PTH hypersensitive phenotype in vitro, at least with respect to c-fos induction since this AP-1 protein is significant to the phenotype of both cells (Wagner, 2010; Boyle, 2003). Recent investigations with c-fos null mice support the hypothesis that the interaction between immature osteoblasts and immature osteoclasts is required for maximum response to intermittent PTH (Luiz de Freitas et al., 2009; Zhao et al., 2006; Koh et al., 2006). Hormone may activate both forward and reverse EphrinB-EphB4 signaling between these cells resulting in the enhancement of bone formation and the suppression of resorption (Luiz de Freitas et al., 2009). This is compatible with the observed increased expression of EphB4 in the null mice (Childress et al., 2011); it is also consistent with the significance of c-fos to the regulation of the ephrins in osteoclasts (Irie et al., 2009). Interestingly, the mRNA expression of PTHR1 in both the WT and KO clonal cells was significantly lower than the levels typically observed in the mature primary osteoblasts during the first 10 days in culture. This suggests that our clones may exhibit an extended immature osteoblast phenotype in culture (Allan et al., 2008); however they ultimately acquire the mature phenotype as evidenced by their mineralizing capacity after 20 days in culture. A more detailed analysis of the phenotype progression of these clonal cells is currently underway.

Our *Nmp4*-KO clonal cell line mimicked the enhanced BMP2-induced alkaline phosphatase expression observed in the primary null osteoblast cultures and these results are consistent with earlier experiments showing a suppression of BMP2-induced alkaline phosphatase in MC3T3-E1 cells over-expressing *Nmp4* (Shen et al., 2002). We observed no difference in the expression of the BMP2 receptor ALK3 between WT and KO cells that could explain the hypersensitivity of the null osteoblasts. The earlier over-expression study indicated that *Nmp4* suppresses BMP2-induced transcriptional activity of the receptor regulated SMADS (R-SMADs), SMAD1 and SMAD5 (Shen et al., 2002) but it is not clear whether the underlying mechanism involves the direct repression of transcription or whether *Nmp4*

interferes with the phosphorylation of the SMADS. Although both the WT and KO clonal lines exhibited a 2.5–3-fold enhanced expression of ALK3 mRNA as compared to the primary osteoblasts, the significance of this difference is not yet clear.

Remarkably, *Nmp4* regulation of BMP2-induced alkaline phosphatase expression in both primary and clonal cells was sensitive to the length of time cells were maintained in culture. The primary KO cells exhibited the enhanced sensitivity to BMP2 after 8 days in culture and 3 days of exposure to growth hormone but lost this acute responsiveness after 15 days in culture and 10 days challenge with growth factor. BMSCs typically proceed through a well-defined developmental differentiation program (Lian et al., 2006; Huang et al., 2004) and therefore our data suggest that *Nmp4* controls BMP2 response in less mature osteoblasts. Interestingly, the null clonal line exhibited acute BMP2 responsiveness after 12–13 days in culture and 10 days exposure to growth factor but not at 5–6 days in culture and 3 days challenge with BMP2. This difference in time course from the primary cells is likely the result of the immortalization and thus alteration of phenotype progression. Further studies are required to elucidate *Nmp4* control of BMP2 response and its dependence on osteoblast phenotype.

Nmp4 is a significant anabolic antagonist that governs the molecular signaling and transcription pathways in multiple cell types (Childress et al., 2010). To disentangle the specific contribution of various cell types to the *Nmp4*-KO bone phenotype will require the use of cost-effective in vitro cell preparations capable of maintaining an authentic phenotype for multiple passages. The mTERT immortalized cells described in this study will be a useful experimental tool for these investigations.

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Figure 1.

The transfection of BMSCs with the catalytic subunit of murine telomerase was successfully employed for preparing immortalized clones from both WT and *Nmp4*-KO mice. (A) Schematic of retroviral vector pBabe-mTert-puro. The functional domains of this vector include MuMoLV LTR, Moloney Murine Leukemia virus long terminal repeat; Ψ^+ , packaging signal; mTert, catalytic subunit of murine telomerase; SV40e/p SV40 enhancer-promoter; and Puro^r, the puromycin resistance gene. (B) Results from the preliminary screening demonstrated that the primary WT and KO osteoblasts (OB, P₀) and the immortalized WT and *Nmp4*-KO clones (P_{18–26}) exhibited similar alkaline phosphatase and von Kossa (mineralization) staining. Alkaline phosphatase staining was performed on cells that had been maintained in culture for 14 days post-seeding supplemented with ascorbic acid (50 μ g/ml). Mineral deposition was assessed on cells that had been maintained in culture for 21 days post-seeding supplemented with 50 μ g/ml ascorbic acid 10nM beta-glycerophosphate.

Figure 2.

The clonal cell lines derived from WT and KO mice constitutively express *mTERT* over a 6 month period in culture. (A) PCR analysis of DNA from primary osteoblasts (OB) and immortalized clones demonstrate that the KO cells lack the *Nmp4* gene. *Neo* (neomycin) was used to replace the *Nmp4* gene and the internal control (IC) is *Rapsn*, receptor-associated protein of the synapse (Robling et al., 2009). (B) RT-PCR analysis of RNA harvested from both primary osteoblasts and immortalized clones showed that *mTERT* expression was equally expressed in the primary cells and clones at the early passages (P₀, primary OBs; P₁₀₋₁₃, clones). (C) RT-PCR analysis of RNA harvested from the clones after several serial passages (P₁₈₋₂₆) demonstrated that *mTERT* expression did not decline but did decrease in the primary osteoblasts after four passages. The primary cells had been in culture for nearly 7 wks with ascorbic acid (50 µg/ml) whereas the clones had been in continuous culture for nearly 6 months with ascorbic acid. *Gapdh* expression was used to normalize relative levels of *mTERT* in all analyses.

Figure 3.

The primary WT and KO osteoblasts (OB, P₀), the immortalized WT and *Nmp4*-KO clones (P_{18–26}), and the osteoblast cell line MC3T3-E1 exhibited similar RNA expression profiles for several osteoblast phenotypic genes including *type I collagen (COL1A1)*, *osteopontin*, *osteocalcin*, *osterix*, and *Runx2* while maintained under ascorbic acid. The mouse embryonic NIH3T3 fibroblasts, cultured for 7 days without ascorbic acid, were included as a negative control for osteoblast phenotype and these cells did not express *osterix*, *osteocalcin*, or *PTHRI* but did express *Runx2*. *Gapdh* was used to normalize gene expression.

Figure 4.

RT-PCR analysis of RNA harvested from cells in culture demonstrated that expression of the bone-specific transcription factor *osterix* and the osteoblast secreted protein *osteocalcin* did not decrease in the WT or *Nmp4*-KO immortalized clones with several serial passages but the expression of these genes in the primary osteoblasts decreased after four passages. (A) *Osterix* and *osteocalcin* mRNA expression in primary WT and KO osteoblasts at passage P₀ and P₄. These cells had been in culture for nearly 7 wks with ascorbic acid (50 µg/ml) (B) *Osterix* and *osteocalcin* mRNA expression in the WT clones at passage P₁₀ and P₂₂–P₂₆. (C) *Osterix* and *osteocalcin* mRNA expression in the KO clones at passage P₁₁–P₁₃ and P₁₈–P₂₂. All the clones had been in continuous culture for nearly 6 months with ascorbic acid (50 µg/ml). *Gapdh* expression was used to normalize relative levels of *osterix* and *osteocalcin* in all analyses.

Figure 5.

The proliferation capacity of the clonal lines was significantly enhanced compared to the primary osteoblasts. The log-transformed cell number of WT (A) and *Nmp4*-KO (B) osteoblasts was regressed against the number of hours from initial seeding of the cells. The slopes of the lines representing the growth of the immortalized clones were significantly greater than the slopes of the lines representing primary osteoblast growth rates ($p < 0.001$). There was no significant difference between the growth rates of any of the clonal lines; additionally there was no significant difference between the growth rates of the primary WT and *Nmp4*-KO cells. The population doubling time, determined as described in Materials and Methods, was on average 2.7-fold shorter for the clonal osteoblasts as compared to the primary bone cells. The statistical analysis was performed with an analysis of covariance (ANCOVA), followed by a Tukey's HSD post-hoc test for all pair-wise comparisons and a Dunnett's post-hoc comparison for contrast with the nontransformed cells.

Figure 6.

The immortalized WT and *Nmp4*-KO clones maintained their capacity for differentiating into adipocytes. Cultures of WT and KO clones were treated with an adipogenic cocktail (induced) or vehicle control as described in Materials and Methods. At the end of the treatment both the induced and control cultures were stained with Oil Red O. On average, 50–70% of the cells from the cultures treated with the cocktail exhibited abundant lipid droplets (shown in orange).

Figure 7.

The primary null osteoblasts and the clone Nmp4^{KO7} exhibit a modest but significantly enhanced PTH-induced increase in *c-fos* mRNA expression at 25nM hormone. [A] Cultures of primary WT and Nmp4-KO BMSC-derived osteoblasts or [B] cultures of clones Nmp4^{WT11} and Nmp4^{KO7} were treated with 25nM hPTH(1–34) or vehicle control for 30 min as described in Materials and Method. At the end of the treatment RNA was harvested from both the induced and vehicle control cultures and analyzed for *c-fos* expression using qRT-PCR. The magnitude of the PTH response for WT or KO primary cells or clones was determined by calculating the average fold-increase in *c-fos* expression normalized to its own vehicle control and these values were compared using a t-test (average $\pm$ SD; asterisk, $p > 0.05$). Experiments with the primary cells and the clones were repeated three times each with identical results, i.e. the primary and clonal KO cells responded as shown in two out of the three experiments.

Figure 8.

The primary null osteoblasts and the clone Nmp4^{KO7} exhibit a significantly enhanced BMP2-induced increase in *alkaline phosphatase* mRNA expression. [A] Cultures of primary WT and Nmp4-KO BMSC-derived osteoblasts or [B] cultures of clones Nmp4^{WT11} and Nmp4^{KO7} were treated with 200 ng/ml BMP2 or vehicle; the primary osteoblasts were treated with BMP2 for 3 days before harvest (8 days in culture). The clones were harvested after 10 days exposure to BMP2 (12–13 days in culture). At the end of the treatment RNA was harvested and analyzed for alkaline phosphatase expression using qRT-PCR. The magnitude of the BMP2 response for WT or KO primary cells or clones was determined by calculating the average fold-increase in alkaline phosphatase expression normalized to its own vehicle control and these values were compared using a t-test (average $\pm$ SD; $p > 0.05$). Two experiments were conducted with the clones and yielded similar results; two of three experiments showed an enhanced KO response to BMP2 with the primary cells. [C] Both the WT and KO clones exhibited on average a 2.5–3-fold enhanced expression of the type I

BMP receptor ALK3. ALK3 expression was normalized to WT primary cells maintained in vehicle for 8 days (average $\pm$ SD; $p > 0.05$).

TABLE I

Gene	Primer sequences for RT-PCR mRNA profile (forward; reverse)	Product size	T _M (°C)
<i>mTert</i> :	5'-CGAAACACACAGACTGCAGAGC-3'; 5'-AGGGTAAAGCTGGTGGAGGTT-3'	383	64
<i>Osteocalcin</i> :	5'-CAAGTCCCACACAGCAGCTT-3'; 5'-AAAGCCGAGCTGCCAGAGTT-3'	371	64
<i>Osteopontin</i> :	5'-CGACGATGATGACGATGATGAT-3'; 5'-CTGGCTTTGGAACTTGCTTGAC-3'	495	67
<i>Colla1</i> :	5'-TTTGTGGACCTCCGGCTC-3'; 5'-AAGCAGAGCACTCGCCCT-3'	226	65
<i>Runx2</i> :	5'-AGCTTCTGTCTGTGCCTTCT-3'; 5'-CCGGGAATGATGAGAACTAC-3'	229	61
<i>Gapdh</i> :	5'-ACCCAGTCCATGCCATCAG-3'; 5'-TCCACCACCCCTGTGTCTGTA-3'	452	66
<i>PTHrP</i> :	5'-GCACACAGCAGCCAAACATAA-3'; 5'-CGCAGCATATAACGACAGGAA-3'	531	65
<i>Osterix</i> :	5'-CTGGGAAAGGAGGCACAAAGAA-3'; 5'-GGGTTAAGGGGAGCAAAAGTCAGGAT-3'	496	68
Gene	Primer sequences for PCR genotype analysis (forward; reverse)	Product size	T _M (°C)
<i>Nrnp4</i> :	5'-CGGGTTTGCTCTTCTCTGAAG-3'; 5'-CCTCCAGACTGGGGTACTGA-3'	351	64
<i>Neo</i> :	5'-AGGATCTCTGTCTCATCTCACCTTGCTCTCTG-3'; 5'-AAGAACTCGTCAAGAAAGGCG-3'	500	75
<i>Rapsy</i> Internal Control:	5'-AGGACTGGGTGGCTTCCAACT-3'; 5'-AGCTTCTCATTTGCTGCGGCCAGGTTCAAGG-3'	575	79
Primers used for sequencing			
pBabe5'	5'-CTTTATCCAGCCCTCAC-3'		55
pBabe3'	5'-ACCCCTAACTGACACACATTC-3'		60
mTERT 1	5'-TTCGAAACAGCAAAACCAACAG-3'		64
mTERT 2	5'-AGGGTAAAGCTGGTGGAGGTT-3'		64