p62 is required for stem cell/progenitor retention through inhibition of IKK/NF-κB/Ccl4 signaling at the bone marrow macrophage-osteoblast niche

Marcus Watkins  
*Washington University School of Medicine in St. Louis*

Roberto Civitelli  
*Washington University School of Medicine in St. Louis*

et al

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Please let us know how this document benefits you.

Recommended Citation

Watkins, Marcus; Civitelli, Roberto; and et al, "p62 is required for stem cell/progenitor retention through inhibition of IKK/NF-κB/Ccl4 signaling at the bone marrow macrophage-osteoblast niche." Cell Reports. 9, 6. 2084-2097. (2014).  
https://digitalcommons.wustl.edu/open_access_pubs/3631

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
p62 Is Required for Stem Cell/Progenitor Retention through Inhibition of IKK/NF-κB/Ccl4 Signaling at the Bone Marrow Macrophage-Osteoblast Niche

Graphical Abstract

Highlights
- Macrophages activate osteoblastic NF-κB, resulting in osteopenia and HSC/P egress
- Autophagic p62 negatively regulates osteoblastic NF-κB activation at several levels
- Nbr1 deficiency rescues the bone and HSC/P egress associated to p62 deficiency

Authors
Kyung Hee Chang, Amitava Sengupta, ..., Jorge Moscat, Jose A. Cancelas

Correspondence
jose.cancelas@uc.edu

In Brief
Chang et al. describe crosstalk between macrophages and osteoblasts that regulates bone formation and hematopoietic progenitor retention and homing to the marrow cavity. The authors find that p62 is required to maintain macrophage-dependent, osteoblast NF-κB repression, osteogenesis, and hematopoietic stem cell/progenitor trafficking.
p62 Is Required for Stem Cell/Progenitor Retention through Inhibition of IKK/NF-κB/Ccl4 Signaling at the Bone Marrow Macrophage-Osteoblast Niche

Kyung Hee Chang,1,2,6 Amitava Sengupta,1,3,6 Ramesh C. Nayak,1 Angeles Duran,4 Sang Jun Lee,2 Ronald G. Pratt,5 Ashley M. Wellendorf,1 Sarah E. Hill,2 Marcus Watkins,6 Daniel Gonzalez-Nieto,1,7 Bruce J. Aronow,8 Daniel T. Starczynowski,1 Roberto Civitelli,3 Maria T. Diaz-Meco,4 Jorge Moscat,4 and Jose A. Cancelas1,2,*

1Division of Experimental Hematology and Cancer Biology, Cincinnati Children’s Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
2Hoxworth Blood Center, University of Cincinnati College of Medicine, 3130 Highland Avenue, Cincinnati, OH 45267, USA
3Stem Cell and Leukemia Lab, Cancer Biology and Inflammatory Disorder Division, CSIR-Indian Institute of Chemical Biology, 4, Raja SC Mullick Road, Kolkata 700032, West Bengal, India
4Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA
5Imaging Research Center, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229, USA
6Division of Bone and Mineral Diseases, Departments of Internal Medicine and Cell Biology and Physiology, Washington University School of Medicine, One Brookings Drive, St. Louis, MO 63110, USA
7Bioengineering and Telemedicine Group, Center for Biomedical Technology, Universidad-Politécnica de Madrid, Pozuelo de Alarcon 28223, Spain
8Biomedical Informatics, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229, USA
9Co-first author
*Correspondence: jose.cancelas@uc.edu
http://dx.doi.org/10.1016/j.celrep.2014.11.031
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

In the bone marrow (BM), hematopoietic progenitors (HPs) reside in specific anatomical niches near osteoblasts (Obs), macrophages (MΦs), and other cells forming the BM microenvironment. A connection between immunosurveillance and traffic of HP has been demonstrated, but the regulatory signals that instruct the immune regulation of HP circulation are unknown. We discovered that the BM microenvironment deficiency of p62, an autophagy regulator and signal organizer, results in loss of autophagic repression of macrophage contact-dependent activation of Ob NF-κB signaling. Consequently, Ob p62-deficient mice lose bone, Ob Ccl4 expression, and HP chemo taxis toward Cxcl12, resulting in egress of short-term hematopoietic stem cells and myeloid progenitors. Finally, Ccl4 expression and myeloid progenitor egress are reversed by deficiency of the p62 PB1-binding partner Nbr1. A functional “MΦ-Ob niche” is required for myeloid progenitor/short-term stem cell retention, in which Ob p62 is required to maintain NF-κB signaling repression, osteogenesis, and BM progenitor retention.

INTRODUCTION

Steady-state blood formation during most adulthood depends on long-lived hematopoietic progenitors (HPs) (Sun et al., 2014). Constitutive egress of bone marrow (BM)-resident HP into the blood is a well-established phenomenon. Circulating HP can survey peripheral organs and foster the local production of tissue-resident innate immune cells under both steady-state conditions and in response to inflammatory signals (Balridge et al., 2010; Essers et al., 2009; Massberg et al., 2007). Dysregulation of stromal components of the HP niches within the BM, such as changes in the levels of chemokines from osteoblasts (Ob) and other mesenchymal cells, has been associated with HP egress (Ding and Morrison, 2013; Greenbaum et al., 2013; Méndez-Ferrer et al., 2010; Omatu et al., 2010; Petit et al., 2002; Sugiyama et al., 2006; Visnjic et al., 2004). Specifically, the deletion of the major hematopoietic stem cell and progenitor (HSC/P) traffic regulator Cxcl12 (Peled et al., 1999, 2000) from Cxcl12-abundant reticular cells and Ob results in constitutive HP mobilization and a loss of B-lymphoid progenitors, whereas their HSC function is normal (Greenbaum et al., 2013). Physiological regulation of these mesenchymal components modulates HP trafficking and is afforded by several mechanisms, including signals derived from BM-resident macrophages (MΦs) (Casanova-Acebes et al., 2013; Chow et al., 2011; Christopher et al., 2011; Winkler et al., 2010). Cellular crosstalk between MΦs and Obs in the HP niche may critically regulate the response of HP to cytokines and chemokines.

The transcription factor NF-κB has a key role in inflammation and immune responses (Ghosh and Karin, 2002; Silverman and Maniatis, 2001; Sun et al., 2013) and has been recently shown to play a role in the response of myeloid progenitors to stress hematopoiesis (Zhao et al., 2014). NF-κB can also control mesenchymal-derived osteogenesis, and mice with a loss of function of NF-κB signaling show osteopenosis (Lotsova et al., 1997). NF-κB
kinase (IKK)-dependent NF-κB activation is essential for the bone-remodeling function of osteoclasts (Ruocco et al., 2005), and the restoration of NF-κB in IKK-deficient mice prevents Ob differentiation (Chang et al., 2009). However, the mechanisms and regulatory pathways that control NF-κB activation in the BM Ob niche and the putative effect of NF-κB signaling on HP activity in the BM remain unknown.

p62 (also called Sqstm-1) is a master regulator of ubiquitinated protein turnover via autophagy and the ubiquitin-proteasome system (Moscat and Diaz-Meco, 2009). p62 also has a central role in osteoclastogenesis. It controls the receptor activator of NF-κB signaling by interacting with TRAF6 and activating NF-κB through atypical-protein-kinase-C-mediated activation of IKK in osteoclasts (Duran et al., 2008; Durán et al., 2004). The loss of p62 signaling is implicated in osteolytic lesions in multiple myeloma and adipogenesis (Hiruma et al., 2009; Rodríguez et al., 2006), whereas gain-of-function mutations of p62 are associated with aberrant and excessive bone turnover in Paget disease (Rodriguez et al., 2006). p62 has also been implicated in the selective autophagy of components of the NF-κB-signaling pathway (Chang et al., 2013); however, the specific cellular and molecular roles of p62 in Ob, and its role in the Ob control of HP activity, have not yet been elucidated. In this manuscript, we reveal that upstream BM-Mφ signaling and cell-to-cell interaction are required for Ob differentiation and the expression of the chemokine Cc14 (Mφ inflammatory protein-1) (H). Whereas the cell-autonomous deficiency of p62 does not translate into significant HP activity defects, the deficiency of p62 in the nonhematopoietic compartment of BM results in osteopenia due to defective Ob differentiation and HP egress. Mechanistically, the p62 within Obb attenuates NF-κB signaling through the downregulation of phospho-focal adhesion kinase (p-FAK), NF-κB, and p-IκBα, thus impairing NF-κB activation, Mφ-dependent Ob differentiation, and Cc14 production.

RESULTS

Deficiency of p62 Induces Non-Cell-Autonomous HP Egress In Vivo

p62−/− mice exhibit egress of myeloid HP (Figure 1A) and short-term (ST) repopulating stem cells (Figures 1B and 1C) to the peripheral blood (PB), but not long- or medium-term repopulating HSCs (Figures S1A–S1C), common lymphoid progenitors (Figure S1D), or B cell lineage populations (Figure S1E). This egress of myeloid progenitors was also observed in the spleen (Figure 1D). However, p62 deficiency was not associated with expansion of the BM content of cells (data not shown); repopulating HSC (Figures S1F–S1H), myeloid, or common lymphoid progenitors or B cell lineage populations (Figures S1I–S1K); or changes in the hematopoietic regenerative response to 5-fluorouracil administration (Figures S1L and S1M).

To determine whether the myeloid progenitor egress is hematopoietic intrinsic or if it depends on the hematopoietic microenvironment (HM), we generated full chimeric animals of wild-type hematopoiesis and p62-deficient hematopoiesis (H-p62−/−) by BM transplantation into CD45.1+ animals (Figure S2A) or through reverse transplantation of WT HSC into lethally irradiated CD45.2+ WT (WT HM) or p62−/− animals (p62−/− HM; Figure 1E). We observed that the effect of p62 on HP traffic is non-cell-autonomous, because H-p62−/− HM did not recapitulate the increased egress of HP (Figure S2B). Conversely, mice lacking p62 in the HM (p62−/− HM) did phenocopy the HP egress of primary mice (Figure 1F), and increased HP egress of p62−/− HM mice was rescued by secondary transplantation into WT recipients (Figures 1G and 1H). All together, these data indicate that the effect of p62 deficiency on HP egress is non-cell-autonomous.

It has been reported that overexpression of AAAGUGG seed-containing microRNA promotes cell expansion, replating capacity, and signaling in hematopoietic cells by interfering with p62-regulated pathways in myeloid cell lines and that these changes may reflect the effect of p62 deficiency on HSC and myeloid progenitor mobilization (Meenhuis et al., 2011). To identify whether p62 regulates in vivo hematopoietic cell proliferation, we analyzed the cell cycle status of primary WT and p62-deficient as well as WT hematopoietic cells engrafted in full chimeric WT HM and p62−/− HM mice. We found no significant differences in the cell cycle status of BM long-term-HSC (Figure S2C), ST-HSC (Figure S2D), and Lin−c-kit+/Sca-1− (LK) (Figure S2E) cells from primary mice or in WT HM or p62−/− HM mice (Figures S2F–S2H). Our data using models of primary loss of function of p62 did not support the existence of a cell-autonomous or microenvironment-dependent role of p62 in HSC/P cell cycle regulation.

To determine whether a defect in the homing of circulating HP is responsible for HP egress in p62-deficient HM, we measured the ability of WT HSC and HP to home into nonmyeloablated WT HM or p62−/− HM. As compared to WT HM recipients, there was a 50%–60% reduction in the homing of immunophenotypically defined BM HSC (Lin−/c-kit+/Sca-1−/CD34+/CD135− cells) to p62−/− HM-recipient mice (Figure 1I) and the progenitor-containing population of Lin−/c-kit+/Sca-1− (LSK) BM cells (Figure 1J), as well as LK BM cells (Figure 1K). This homing defect suggested a significant impairment in the ability of the p62−/− HM to lodge HSC/P upon transplantation into an unmanipulated host. Nevertheless, this difference in HSC/P homing ability disappeared when the recipient mice were myeloablated following lethal irradiation (Figures S2I–S2K), suggesting the existence of an accessory contribution from a radiosensitive cell population that can be regenerated by WT HSC/P transplantation.

Ob Deficiency of p62 Is Responsible for Osteogenic Defects and HP Egress

The mammalian BM microenvironment consists of multiple cell types, including mesenchymal progenitors, CXCL12-expressing adventitial reticular cells, Ob lineage cells, endothelial cells, pericytes, fibroblasts, and adipocytes, among others. Functional studies support a role for Ob-lineage cells to maintain HP in the BM in vivo. Long-term HM are connected to Ob (Zhang et al., 2003). Ob in the BM contribute to HP expansion through activation of Notch signaling (Calvi et al., 2003), and targeted ablation of Ob in vivo results in a loss of HP from the BM (Visnjic et al., 2004). To understand whether osteogenesis was impaired in p62−/− HM mice and to delineate the role of p62 in the nonhematopoietic HP niche, we examined the bone architecture of p62−/− HM chimeric mice. Bone histomorphometric analysis using microcomputed tomography (micro-CT)
Figure 2. p62 Regulates HP Trafficking in a Non-Cell-Autonomous Manner
(A) CFU-C content in the PB (PB) of WT or p62−/− mice (n = 8–10 mice per group). Values represent average of three independent experiments.
(B) Experimental set up. PB mononuclear cells (PBMC) from CD45.2+ WT or p62−/− mice were mixed with CD45.1+ B6.SJLPpcrePep3bBoyJ WT bone marrow (BM) cells and competitively transplanted into lethally irradiated CD45.1+ B6.SJLPpcrePep3bBoyJ mice.
(C) CD45.2+ chimera in the PB of recipient mice (n = 5–8 mice per group) after 6 weeks of competitive transplantation.
(D) Frequency of hematopoietic progenitors in the spleens of WT or p62−/− mice (n = 3 mice per group).
(E) Experimental setup. BM cells from CD45.1+ B6.SJLPpcrePep3bBoyJ mice were noncompetitively transplanted into lethally irradiated CD45.2+ WT or p62−/− mice to generate chimeric WT HM or p62−/−HM mice.
(F) Absolute numbers of CFU-C present in the PB of WT HM or p62−/− HM mice (n = 4–7 mice per group).
(G) Experimental setup. BM cells from CD45.1+ B6.SJLPpcrePep3bBoyJ mice were noncompetitively transplanted into lethally irradiated CD45.2+ WT or p62−/−HM mice. BM cells isolated from primary WT HM or p62−/−HM mice were transplanted into lethally irradiated CD45.2+ to generate secondary WT recipients.
(H) Absolute numbers of CFU-C present in the PB of secondary WT-recipient mice (n = 4–6 mice per group).
(I–K) Homing (%) of WT ST-HSC (I), LSK (J), and LK (K) BM cells to nonmyeloablated BM from WT or p62−/− mice. A minimum of five mice were analyzed per group. For all panels, values represent mean ± SEM. *p < 0.05.

(Figure 2A) revealed significantly decreased femoral trabecular bone volume (Figure 2B) and trabecular number (Figure 2C) in p62−/− HM mice as compared to WT HM mice. In p62−/− deficient femoral sections, histological analysis of endosteal Col1α1-expressing Ob demonstrated a flatter appearance (Figures 2D and 2E) and a decreased number of cortical osteocytes (Figure 2F). However, the frequency (and content) of mesenchymal progenitors (colony-forming unit fibroblasts [CFU-F]; Figure 2G) and osteoprogenitors (CFU-Ob; Figure 2H) did not change, suggesting that p62 controls Ob maturation and terminal differentiation, but not mesenchymal progenitor differentiation. Finally, to confirm whether the presence of p62 in Ots is responsible for HP mobilization, we enumerated the number of circulating HP in mice with a specific deletion of p62 in their Ots. We crossed Col1α1(Cre) mice with p62F5 mice to obtain Ob-specific Col1α1(Cre);WT and Col1α1(Cre);p62F5 mice. Interestingly, Col1α1(Cre);p62F5 mice phenocopied increased HP egress as observed in p62−/− and p62−/−HM mice (Figures 2I and 2J), suggesting that the loss of p62 in Ots is responsible for non-cell-autonomous HP egress in vivo.

p62 Regulates NF-κB-Dependent Ob Differentiation in Presence of Mfs
The homing defect that is associated with a deficiency of p62 in nonconditioned HM (Figures 1H and S2I-S2K) is not found in mice with irradiated HM; this may suggest that radiosensitive hematopoietic cells are required as effectors to retain HP in the BM. Myeloid mononuclear accessory cells, in addition to Ots, have...
been proposed to form a myeloid-signaling network responsible for HP homing and engraftment in the BM (Katsumoto et al., 2005). Specifically, Møs have been known to mediate HP retention in the BM (Casanova-Acebes et al., 2013; Chow et al., 2011). It has been shown that depletion of BM Møs, but not other lineage-related cells such as osteoclasts (Miyamoto et al., 2011), is adequate to suppress endosteal Obs, inhibit the expression of HP-supportive cytokines at the endosteum, and elicit HP mobilization into the PB (Winkler et al., 2010). The content of Møs in contact with Obs in the trabecular and endosteal lining of BM from WT HM and p62−/− HM mice was similar (Figures S3A and S3B). Similar to untreated mice with global deficiency of p62 (Durán et al., 2004), the loss of p62 in Obs did not modify significantly the bone osteoclast content (Figures S3C and S3D). Together, these data suggested that the content of osteogenic Møs or osteoclasts was not causative of the loss of BM retention observed in HM- or Ob-p62-deficient mice.

To identify whether Møs are required for p62-mediated Ob activity, we isolated and expanded F4/80+/CD68+/CD115+/CD169− Møs derived from the BM of ubiquitin C-enhanced GFP (EGFP) mice (Figure S3E) and cocultured with Obs in vitro at a 1:1 ratio for 24 hr in the absence of Møs or osteogenic differentiation factors and then analyzed their biochemical and expression effect on downstream signaling. Notably, the expression levels of Ob differentiation genes are significantly reduced in p62−/− mice (Figure S3F). The absence of p62 expression in Obs resulted in a modest reduction of expression of p62−/− mice (Figure S3F). The absence of p62 expression in Obs resulted in a modest reduction of expression of p62−/− cells, but not other lineages.

Figure 2. Ob p62 Deficiency Impairs Ob Differentiation and Osteogenesis In Vivo, Resulting in HP Egress

(A) Representative microcomputer tomography (micro-CT) analyses of femoral trabecular bone of WT HM or p62−/− HM mice after 16 weeks of transplantation. (B and C) Percent ratio of trabecular bone volume versus tissue volume (BV/TV) and trabecular number (Tb.N) of WT HM or p62−/− HM mice (4–7 mice per group) at 16 weeks after transplantation. (D) Representative confocal microscopic images of collagen type1α1 (red) and nuclear counterstaining (DAPI, blue) in femur sections from WT HM or p62−/− HM. Analysis was performed as measurement of the transversal diameter at the widest point of the Obs expressing Col1α1 (space between arrow ends). (E) Measurement of Ob length projections of bone lining Obs in longitudinal femoral sections from WT HM (Obs n = 24) or p62−/− HM (Obs n > 17 per group). *p < 0.05. (F and H) CFU-F and CFU-Ob from BMNCs of chimeric WT HM or HM p62−/− mice. (G) Counts of osteocytes in femoral cortical bone from WT HM (n = 47 fields) or p62−/− HM (n = 42 fields). (H) CFU-Ob/5x10⁶ BMNC from Col1α1-Cre; WT or Col1α1-Cre; p62−/−, n = 33 fields were analyzed for each group. For all panels, values represent mean ± SEM. ***p < 0.001.
Extracellular signaling-regulated kinase (Erk) or p38 activation has been shown to be required for Ob differentiation. The Erk or p38 pathway mediates the signal transduction from hormone and growth factors, such as fibroblast growth factor-2 (Xiao et al., 2002) and parathyroid hormone (Chen et al., 2004). They also stimulate Runx-2 phosphorylation and its transcriptional activity (Franceschi et al., 2009; Xiao et al., 2000). In addition, Erk or p38 signaling is required for p62 activity in other mesenchymal lineage BM cells such as adipocytes. It was reported that the absence of Erk1 was sufficient to restore abrogated adipogenesis and energy homeostasis of p62-deficient animals in vivo (Lee et al., 2010). However, whereas Mφ signaling is associated with increased activation of Erk, but not p38, in fluorescence-activated cell sorting (FACS)-sorted Obs (Figure S3G), the deficiency of p62 resulted in inhibition (Figure S3G) rather than further upregulation as was reported in adipocytes (Lee et al., 2010; Rodriguez et al., 2006). Furthermore, HP egress was not rescued by deletion of Erk1 (Figure S3H), strongly suggesting that Erk upregulation is not responsible for HP mobilization in p62-deficient Obs.

NF-κB signaling has also been shown to be crucial in Ob differentiation. Mice with disrupted NF-κB signaling show severe osteopetrosis (Iotsova et al., 1997). IKK-dependent NF-κB activation is essential for the bone remodeling function of osteoclasts (Ruocco et al., 2005), and gain of function of NF-κB activity prevents Ob differentiation, as demonstrated in mice deficient in IKK (Chang et al., 2009). Because osteoclasts may be
dispensable for HP mobilization in vivo (Miyamoto et al., 2011), we analyzed whether NF-κB-dependent signaling in Obs correlates with HP egress. Histological analysis of active NF-κB p65 in endosteal and trabecular Obs showed increased NF-κB nuclear localization in p62−/− Obs in vivo (Figures 3B and 3C). Similar to previous reports in osteoclasts and tumor cells (Duran et al., 2008; Durán et al., 2004), Obs cultured from p62-deficient animals showed decreased levels of the p65 subunit of NF-κB bound to DNA (Figure 3D), and IkBα, the phosphorylated inhibitor of NF-κB (p-IkBα; Figure 3E), was responsible for degradation. However, cocultures of MΦs with Obs resulted in increased NF-κB p65 activity, as demonstrated by translocation of NF-κB to the nucleus (Figure 3F). In addition, p62−/− Obs isolated after coculture with MΦs showed increased phosphorylation and expression of IkBα (Figure 3G).

Altogether, these data demonstrate that MΦs reverse p62-dependent NF-κB signaling in Obs. To ascertain whether NF-κB is crucial for p62-dependent Ob differentiation, we evaluated the changes of bone-specific gene expression after in vitro treatment with BAY 11–7085, an NF-κB inhibitor. The attenuation of NF-κB activity was validated by the reduced concentration of DNA-bound-NF-κB p65 seen in both Obs and MΦs (Figures S3I and S3J). Interestingly, addition of BAY 11–7085 to Ob-MΦ cocultures derepressed Alpl, Runx2, and Bglap expression in WT and p62−/−−/−sorted Obs (Figures 3H–3J), suggesting that p62 deficiency enhances the ability of IKK/NF-κB activity to inhibit Ob differentiation. Finally, Cxcl12-driven chemotaxis in the presence of WT MΦ-p62-deficient Ob cocultures was significantly reduced (Figure 3K), indicating that Ob p62 deficiency results in a loss of sensitivity to Cxcl12 chemotaxis gradients. The inhibition of NF-κB in WT MΦ-WT Ob cocultures results in an ~2-fold increase in chemotaxis of WT HP and eliminates the effect of p62 deficiency in Obs, suggesting that NF-κB activity is responsible for the effect of Ob p62 on directed migration toward Cxcl12 gradients (Figure 3K).

HM p62 Deficiency Impairs MΦ-Dependent Ob Expression of the Chemokine Ccl4 and Recapitulates Part of the Mobilization Phenotype Associated with BM MΦ Activity

In order to determine whether the osteogenic deficiency of p62 in p62−/− HM mice could result in the alteration of the levels of chemokines responsible for HP egress, we performed an in vivo expression screen of a panel of chemokines or cytokines known to be relevant in HP traffic (Figures S4A–S4N). We hypothesized that their expression and secretion in plasma and/or femoral extracellular fluid may depend on Ob activity, which could be regulated by NF-κB activity.

Interestingly, Ccl4 was uniquely found to be downregulated in the femoral extracellular fluid of p62-deficient mice (Figure 4A) and trended to be decreased in plasma as well (Figure S4A). Ccl4 is a C-C motif chemokine that modulates BM HP chemotactic response to Cxcl12 (Basu and Bromley, 2009). Cxcl12 levels in the plasma and bones of p62−/− HM mice were normal or upregulated (Figures S5A and S5B), which was consistent with a null or minimal contribution of mature Obs to systemic or local Cxcl12 levels, as previously reported (Ding and Morrison, 2013; Greenbaum et al., 2013). As expected, Obs from WT mice did not show any significant expression or secretion of Ccl4; neither did the deficiency of p62 modify its expression or secretion (Figures 4B–4D). Interestingly, the expression and secretion of WT Ob Ccl4 protein was significantly increased upon coculture with MΦs, whereas the deficiency of p62 in Obs resulted in diminished levels of Ccl4 expression and secretion, similar to the levels of unstimulated Obs (Figures 4E–4G, cf. Figures 4B–4D).

Administration of clodronate liposomes has been shown to deplete MΦs and induce HP mobilization (Winkler et al., 2010). We compared the effect of MΦ depletion by clodronate with the deficiency of Ob p62, in terms of their effect on HP mobilization. We found that clodronate did result in a depletion of CD11b+/F4/80+/CD68+/CD115 (c-fms)+ MΦs in vivo and did not affect other CD11b+/F4/80+ BM cell populations (Figure S5C). Exhaustion of CD11b+/F4/80+/CD68+/CD115+ induced an ~4-fold increase in the number of circulating HP whereas Ob p62 deficiency resulted in only an ~2-fold increase (Figure S5D). Interestingly, the effect of HM p62 deficiency on HP mobilization was lost in CD11b+/F4/80+/CD68+/CD115− depleted animals (Figure S5D). These results indicate that CD11b+/F4/80+/CD68+/CD115+ MΦs signal through Ob p62 and that Ob p62 is, at least partly, responsible for the effect of CD11b+/F4/80+/CD68+/CD115+ cell depletion on myeloid progenitor retention in the BM.

We confirmed that direct cell-to-cell interaction between Obs and MΦs was necessary to modulate Ccl4 production, because culture of Obs and MΦs in different chambers of a noncontact transwell culture system failed to reproduce the difference in expression of Ccl4 (Figure 4H). Moreover, addition of an anti-Ccl4 antibody to cocultures of p62-deficient Obs and WT MΦs restored the ability of WT HP to respond to Cxcl12-driven chemotaxis (Figure 4I). Collectively, our data indicate that p62 prevents activation of IKK and NF-κB in Obs and that p62 is required for osteogenesis, Ob differentiation, expression of the chemokine Ccl4, Cxcl12-directed chemotaxis, and HP retention within the BM microenvironment.

p62 Signaling Attenuates IKK/NF-κB Activity through Its Autophagic Activity, Facilitating Ob Differentiation and Ccl4 Expression

To mechanistically ascertain the role of p62 in Ob differentiation and Ob Ccl4 expression, p62−/− Obs were transduced with a retroviral EGFP-expressing bicistronic vector expressing full-length p62 or an empty vector (mock; Figure S6A). Transduced cells were cocultured (or not) with WT (unlabeled or ubiquitin C-EGFP transgenic) MΦs for 24 hr. This experimental setting was used to determine the effect of the restoration of p62 expression on NF-κB activity and signaling pathway. As a positive control of optimal NF-κB translocation, we used stimulation with tumor necrosis factor alpha on Obs (Figure S6B). We confirmed that MΦ signaling was necessary for p62-dependent NF-κB nuclear translocation, because exogenous expression of p62 did rescue and abrogate the nuclear localization of active NF-κB p65 in p62-deficient Obs in the presence of MΦs (Figure 5A), but not in their absence (Figure S6B). Reintroduction of p62 decreased the cellular levels of NF-κB p65 protein (Figure 5B) due to both lysosomal degradation and a loss of nuclear
translocation, as assessed by colocalization in lysosomes and diminished nuclear translocation (Figures 5A and 5C). Inhibition of NF-κB p65 activation by p62 was secondary to restored catalytic activity of IKK activity, given that the absolute cytosolic subunits α, β, or γ (Figure S6C), indicating that p62 controls IKK activity, but not its expression.

Similarly, exogenous expression of p62 in p62−/− Obs in the presence of MΦs rescued the expression of Ob Runx2 (Figure 5E), confirming that p62 expression is associated with Ob differentiation. Overexpression of p62 also rescued the expression and production of Ccl4 by Obs (Figures 6A–6C), indicating that the concentration of Ccl4 is directly related to p62 expression and that it is inversely related to the activity of NF-κB in Obs cultured with MΦs.

Protein degradation by p62-dependent autophagy mechanisms may be determined by the oligomerization-dependent autophagosome localization (Komatsu et al., 2007; Itakura and Mizushima, 2011). To identify the functional domain in p62 responsible for Ccl4 production, p62Δ69-73 mutant, which fails to oligomerize, a process required for autophagosome formation (Duran et al., 2008, 2011; Moscat et al., 2006). The chemoattraction of HP toward Cxcl12, which was added to cultures of WT MΦs and WT Obs, was significantly inhibited by the autophagy inhibitor bafilomycin A1 to the same level seen in cultures of WT MΦs and p62Δ69-73 mutant (Figure 6D; compare with Figure 3K). This suggests that the effect of p62 deficiency is equivalent to that induced by bafilomycin A1 through autophagic inhibition. Ectopic expression of the p62 Δ69-73 mutant (Figure S6D), which disrupts the p62 oligomerization required for autophagosome formation, failed to rescue Ccl4 expression or production (Figures 6A–6C), suggesting that p62-dependent Ccl4 expression depends on oligomerization, which is required for localization to the autophagosome formation site.
Together, these data indicate that p62 represses NF-κB signaling and that it is necessary for Ob differentiation and Ccl4 production through its role in autophagy.

Nbr1 Antagonizes p62, and Its Deficiency Rescues BM MΦ-Dependent Ob Ccl4 Expression and HP Retention

Phox bemp1 (PB1)-domain-containing proteins p62 and Nbr1 share domain architecture and play overlapping roles in cell signaling through protein-protein interaction. A C terminus deletion of Nbr1 results in Ob differentiation (Whitehouse et al., 2010). To identify whether Nbr1 has overlapping or distinct roles in osteogenesis and HP retention, we crossed cytomegalovirus (CMV)-Cre; Nbr1 f/f (Nbr1<sup>D/D</sup>) mice (Yang et al., 2010), where Nbr1 is absent in germinal cells, with p62<sup>C0/C0/C0</sup> mice (p62<sup>C0/C0/C0</sup>; Nbr1<sup>D/D</sup>). We analyzed the effect of HM deficiency of full-length Nbr1 and of double Nbr1/p62 on osteogenesis and HP mobilization in chimeric animals. We identified that Obs isolated from p62<sup>C0/C0/C0</sup> bones express increased levels of Nbr1 (Figure 6E), suggesting a compensatory role. The deficiency of Nbr1 in vivo does not significantly impair osteogenesis, Ccl4 production, or levels of circulating HP (Figures 6 F–6J). Surprisingly, the double deletion of Nbr1 in p62<sup>C0/C0/C0</sup> HM mice (p62<sup>C0/C0/C0</sup>; Nbr1<sup>D/D</sup>) ameliorates bone architecture, Ccl4 production, and HP egress, which suggests distinct roles for p62 HM and Nbr1 in hematopoiesis and an antagonistic effect of Nbr1 on p62 activity.

Figure 5. Ectopic Expression of p62 Abrogates Nuclear Translocation of NF-κB p65 and Rescues Differentiation and Ccl4 Expression of p62-Deficient Obs

(A) Representative confocal microscopy images of NF-κB p65 (green), lysosomes (LAMP2, red), and nuclear counterstaining (DAPI, blue) in cocultures of FACSSorted, mock-vector-transduced WT (WT + Mock), p62-deficient (p62<sup>C0/C0/C0</sup> + Mock) or p62-transduced-p62-deficient (p62<sup>C0/C0/C0</sup> + p62) Obs (DAPI, asterisks) cultured with unlabeled MΦs (DAPI; small nuclei with condensed chromatin) for 24 hr.

(B) Representative immunoblot of total NFκB p65 in postculture-isolated Ob lysates from (A). β-actin was used as a loading control.

(C) DNA-bound NFκB p65 in lysates of the nuclear fraction of Ob cells isolated postculture from (B).

(D) Immunoblot of phosphorylated IκBα and total IκBα expression in the cytosolic fraction of cells from (B).

(E) Change (fold) in Runx2 mRNA expression of transduced Obs after 24 hr of coculture with WT MΦs. Values are derived from three independent experiments. For all panels, values represent mean ± SEM. *p < 0.05; **p < 0.01.
DISCUSSION

The circulation of HSC/P in the PB is crucial as part of a system of immunosurveillance of the peripheral organs and to foster the local production of tissue-resident innate immune cells (Massberg et al., 2007). Niche regulation of HP trafficking in vivo is incompletely understood. Cellular and molecular specificity, as well as crosstalk, are important aspects in order to understand how cell signaling within complex networks generates such precise cellular responses to a myriad of different stimuli. A key to this process is intracellular protein scaffolds, which are multidomain proteins that assemble specific signaling complexes in different cellular locations so as to assure a spatially and temporally controlled signal.

The role of Obs in HSC/P traffic remains controversial, and the genetic characterization of the molecular signatures within the Ob microenvironment, which have been implicated in contributing to HSC/P trafficking in vivo, is quite incomplete. Previous experiments have provided insights into the mechanisms by which Ob scaffolds regulate HSC/P trafficking, and these findings have highlighted the importance of understanding the role of Ob scaffolds in the regulation of HSC/P trafficking in vivo.
reports have indicated that Ob activity controls HSC/P retention within the BM (Calvi et al., 2003; Raaijmakers et al., 2010; Visnjic et al., 2004; Zhang et al., 2003), and Mφs that are in contact with Obs (called osteomacs) (Winkler et al., 2010) have been recently implicated as intermediate cells in granulocyte colony-stimulating factor mobilization, Ob depletion, and in HSC/P egress from the BM (Katayama et al., 2006; Winkler et al., 2012). However, modulation of Ob numbers does not necessarily alter HSC numbers (Kiel et al., 2007; Zhu et al., 2007), suggesting that functional changes in Ob activity may be responsible for their effect on BM HSC/P retention. Cxcl12-dependent progenitor retention (Greenbaum et al., 2013) and B lymphopoiesis (Zhu et al., 2007; Greenbaum et al., 2013; Ding and Morrison, 2013) are functional roles that have been assigned to Ob lineage populations of the BM. However, the specific role of Obs in HSC/P mobilization remains controversial (Kiel et al., 2007; Ding and Morrison, 2013).

The deficiency of p62 in the nonhematopoietic compartment of BM results in osteopenia as a result of loss of Ob differentiation and induces egress of ST-HSC and myeloid progenitors. This effect is due to impaired Ob signaling, because the deficiency of p62 in Obs, as identified by the expression of ColIα1 driven by its 2.3 Kb promoter/enhancer, phenocopies the mobilization of HSC/P of mice with p62-deficient HM (Dacquin et al., 2002). However, the deficiency of p62 does not result in either significant mobilization of long- or medium-term repopulating HSC, or in changes in the BM content or mobilization of B lymphopoietic cells, suggesting that p62 regulates some, but not all, of the activities associated with Ob activity in the BM (Winkler et al., 2010; Chow et al., 2011; Zhu et al., 2007; Greenbaum et al., 2013). This mobilization effect results in decreased homing of ST-HSC and LSK/LK progenitors in nonmyeloablated recipients, but not in myeloablated recipients. The effect of myeloablation is restored by transplantation, because p62−/− HM mice display increased numbers of circulating HSC/P after transplantation. We hypothesized that a WT radiosensitive cell population with the ability to be regenerated by 6 weeks posttransplantation may be responsible for this differential effect and may contribute to significant changes in the content or activity of the osteoblastic niche. Several reports have provided information about the functional defects and apoptosis of irradiated Mφs in vivo in C57Bl/6 mice as early as within the first 24 hr after irradiation (Coates et al., 2008; reviewed in Mukherjee et al., 2014). Therefore, tissue Mφs are major candidates in relation to the BM Ob niche. Other radiosensitive populations such as osteoclasts, which have also been reported to control HSC/P traffic in the BM (Kollet et al., 2006), were analyzed in the BM of WT HM and p62−/− HM mice and found not to be regenerated by 6 weeks posttransplantation (data not shown).

A putative effect of p62 on BM HSC/P proliferation (Meenhiusen et al., 2011) as a downstream target of microRNAs 17/20/93/106 was explored. Our data could not confirm changes in HSC/P proliferation, as assessed by flow cytometry analysis of bromodeoxyuridine uptake in vivo, in either primary mice or HM chimeric BM HSC/P. Analysis of p62−/− and p62−/− HM mice for as long as 6 months after birth or transplantation did not show any significant sign of hematopoietic failure (data not shown). Whereas it is possible that long-term aging of p62−/− or p62−/− HM mice results in significant defects in BM HSC/P content, our data focus on the effect of p62 expression on the ST-HSC and myeloid progenitor populations.

This report describes and analyzes a mechanism of Mφ-Ob-dependent HP retention in the BM, in which Mφs regulate Ob differentiation. We explored the existence of functional crosstalk between Mφs and Ob lineage cells that would regulate HSC/P trafficking and identified a three-cell (Mφ; Ob, and HSC/P) interplay of interactions in vivo, which could be recapitulated and mechanistically analyzed in cellular models. Our data provide evidence that (1) cell contact between p62 in Mφs and Obs induces Ob NF-κB activity and differentiation; (2) the maintenance of low levels of Ob NF-κB activity is crucial for Ob differentiation, Cxcl12-directed chemotaxis, BM retention of myeloid progenitors, and the upregulation of NF-κB activity secondary to the loss of p62 results in egression of myeloid progenitors and ST-HSC to the PB; (3) autophagy p62 is a negative regulator of NF-κB activity controlling the levels of p-FAK, p-IkBα, and NF-κB translocation; and (4) Nbr1, a PB1-binding partner of p62, antagonizes p62 activity as a negative regulator, and the loss of Nbr1 rescues the deficient osteogenesis of p62-deficient animals. This signaling pathway is summarized in Figure S6E.

Circulating HP can foster the local production of tissue-resident innate immune cells in response to inflammatory signals (Baldrige et al., 2010; Essers et al., 2009; Massberg et al., 2007). The transcription factor NF-κB has a key role in inflammation and immune responses and has been recently shown to play a role in myeloid progenitor response to stress hematopoiesis (Zhao et al., 2014). Inflammatory signals through NF-κB probably underlie the stress circulation of HSC/P. Mφs are well-known mediators of intrinsic activation of NF-κB activity in response to infection or other inflammatory cues. Here, we demonstrate that Mφ signaling is required for Ob differentiation and Ob NF-κB activation and is crucial for the expression and production of Ob Ccl4, a modulator of Cxcl12 activity that affects HP retention in the BM (Basu and Broxmeyer, 2009). Obs would act as signal amplifiers and coordinators of inflammatory signals initiated by infection-responsive Mφs. Our data reveal Ccl4 as a chemokine expressed and secreted by differentiated Obs in contact with BM Mφs and confirm in vivo previous data on the role of Ccl4 as a modulator of Cxcl12-dependent HSC/P traffic in the BM (Basu and Broxmeyer, 2009).

Obs have high autophagy activity during differentiation (Liu et al., 2013), and NF-κB signaling prevents Ob differentiation (Chang et al., 2009). Oligomerization of p62 is required for autophagosome formation (Itakura and Mizushima, 2011), where p62 aggregates ubiquitinated proteins (Björkoy et al., 2006) and has been shown to regulate the degradation of the RelA component of NF-κB in Obs through selective autophagy (Chang et al., 2013). We have identified that p62 attenuates Mφ-dependent NF-κB signaling in Obs through the downregulation of p-FAK, NF-κB, and p-IκBα, which results in impaired NF-κB activation and Ob differentiation in vivo (Figure S6E). Pharmacological inhibition of NF-κB in Mφ/p62−/− Ob cocultures restored Ob differentiation and Cxcl12-driven chemotaxis, confirming the mechanistic role of NF-κB in the osteogenic defect of p62-deficient mice. Neutralization of Ccl4 and inhibition of autophagy also
restored the deficient Cxcl12-directed chemotaxis seen in the same cocultures, indicating that all are dependent on the same pathway and regulated by p62. Finally, restoration of p62 expression in the Obs represses NF-κB activity to levels similar to WT and induces Ob differentiation, Ccl4 expression, and progenitor retention in vivo. Our data also suggest that, during Ob-Mφ crosstalk, p62 relies on p62-PB1 oligomerization for Ccl4 expression.

BM Mφs are essential to our understanding of Ob activity, osteogenesis, and HP traffic. The interaction between differentiating cell- and niche-derived signals has also been shown to play an important role in Drosophila HP maintenance (Mondal et al., 2011). Clodronate-induced deficiency of Mφs results in progenitor mobilization (Winkler et al., 2010). We have confirmed that the deficiency of CD11b+/F4/80+/CD68+/CD115− BM Mφs does result in a 4-fold increase in myeloid progenitor mobilization, which is ∼2-fold higher than the mobilization observed in p62-deficient animals. Interestingly, the Ob p62 deficiency does not add or synergize with the depletion of Mφs, indicating that the effect of Ob p62 deficiency on myeloid progenitor retention depends on the existence of BM Mφs in vivo. Subpopulations of Mφs may be specifically controlling myeloid progenitor retention. BM CD169+ Mφs have been shown to promote the retention of HSC/P in the mesenchymal stem cell niche (Chow et al., 2011). Our experiments did not specifically address the role of Ob activity in relation to CD169+ Mφs, because the cultured primary Mφs did not express CD169. It is possible that CD169+ BM Mφs share the activities of BM CD169− Mφs in regards to the activation of Ob NF-κB activity and downstream effects; however, this point is unresolved and will require further dissection in vivo of the specific Mφ populations responsible for Ob NF-κB activity.

In our system, the interaction between Obs and Mφs has been exclusively contact dependent as some of the phenotypes, such as upregulation of Ccl4 expression, could not be reproduced in noncontact transwell systems. We provide evidence that p62 regulates osteogenic signals in an Ob-Mφ coculture setup. This signal, probably mediated by integrins, relies, at least in part, on FAK or other redundant family proteins that are responsible for cell-to-cell anchoring. Integrins link the inside of a cell with its outside environment and, in doing so, regulate a wide variety of cell behaviors. Integrins play an important role in angiogenesis and cell migration; however, their functions in bone formation are less clear. The majority of integr signaling proceeds through FAK, an essential component of the focal adhesion complex. The loss of FAK does not perturb Ob differentiation in vitro or in vivo, owing to the compensatory increase in Pyk2 in Obs (Kim et al., 2007). FAK and Pyk2 are substrates of autophagy, and there is emerging evidence implicating autophagy as an important mediator of bone cell function in normal physiology (Hocking et al., 2012) and in pathology as documented by the role of p62 mutations in Paget disease of the bone (Laurin et al., 2002; Rea et al., 2013).

Genetic truncation of Nbr1, a selective autophagic receptor for degradation of ubiquitinated substrates that can interact with p62, but not LC3, leads to increased Ob differentiation and activity in vivo (Whitehouse et al., 2010). As shown by our data, Nbr1 deficiency restores in vivo BM Ccl4 production and HP mobilization, suggesting that it plays an inhibitory role on the p62-dependent regulation of NF-κB activity in Obs.

Our data define a signaling network between BM Mφs and neighboring Obs with activity on the HP niche. We propose the existence of a regulatory signal from BM-resident “osteo-macrophages,” where Ob NF-κB signaling is connected with the immunosurveillance functions of circulatory HP. We also propose the existence of a homeostatic regulatory role for selective autophagy regulated by p62 on the NF-κB-signaling pathway in Obs, which is required for osteogenesis and BM progenitor retention. A connection between bone, innate immunity, and progenitor traffic is proposed, which has the ability to amplify or inhibit Mφ-dependent inflammatory signals. Our data support a role for p62 in the regulation of the intercellular signaling at the BM Mφ-Ob niche and identifies the key molecular determinants of signaling that regulate ST-HSC and myeloid progenitor trafficking.

**EXPERIMENTAL PROCEDURES**

**Mice**

All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols were approved by institutional care and use committees for animal research at the Cincinnati Children’s Hospital Research Foundation. p62+/−; p62−/−; Erk1−/−, and CMV-Cre; Nbr1−/− mice have been described previously (Lee et al., 2010; Rodriguez et al., 2006; Yang et al., 2010). p62−/− mice were crossed with Nbr1−/−a mice to generate p62−/−; Nbr1−/−a mice. Genetically modified Col1a1(2.3 Kb promoter/enhancer)-Cre mice (Dacquin et al., 2002) were crossed with p62−/− mice (Müller et al., 2013) for generation of osteo-lineage-specific deletion of p62. All primary mice were analyzed between 6 and 10 weeks of age. Chimeric (Hm) mice were generated by noncompetitive transplantation of WT BM nucleated cells (BMNCs) from B6.SJL/Fplc/J (Fplc, B6.SJL) into lethally irradiated 6- to 8-week-old CD45.2 WT or p62−/− deficient mice. All mice were maintained in C57Bl/6 back- ground. C57Bl/6 (CD45.2+), and B6.SJL/Fplc/J (CD45.1+). mice were obtained commercially (Jackson Laboratory; Harlan Laboratories). Littermate mice from the same breeding were used in all experiments. Ubiquitin C-EGFP mice have been described previously. These mice had been backcrossed greater than ten generations into C57Bl/6 mice.

**Quantification of HP Egress**

PB total and differential counts were analyzed using a Hemavet 950 (DREW Scientific). PB was isolated by retro-orbital bleeding. Hematopoietic progenitors isolated from BM, spleen, or PB were grown on methylcellulose medium supplemented with cytokine cocktails (Stem Cell Technologies), and colony-forming progenitors (CFU-C) were scored on day 9.

**HSC Repopulation**

Adult recipient mice were lethally irradiated with a Ca377 gamma irradiator as previously described (Sengupta et al., 2011). For competitive repopulation experiments, CD45.2+ BMNCs or PB (50–150 μl) were mixed with 500,000–3 × 10^6 CD45.1+ BMNCs and were transplanted into lethally irradiated B6.SJL/Fplc/J (CD45.1−) recipient mice. HSC engraftment was measured by chimera assessment using flow cytometry at 6 weeks, 10 weeks, and 16 weeks posttransplantation. Chimera level was normalized to WT levels at 100% to allow interexperiment comparison.

**Homing Assays**

For the homing assay to nonmyeloablated BM, 20–25 × 10^6 carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled BM cells from WT mice were prepared and used for injection though the tail vein injection. WT or p62−/− animals were used as recipients and sacrificed after 3 hr posttransplantation. The single-cell suspensions of the BM were subjected to FACS.
analysis to measure homing of HSC (CFSE+Lin−Sca-1+CD45+CD34+), LSK, and LK cells. The percent of homing was calculated from the input and output cell numbers. To measure homing to myeloablated BM, 23 × 10^6 WT BM cells were transplanted into lethally irradiated WT or p62−/−-recipient mice. Sixteen hours after transplant, the recipient mice were sacrificed and the BM cells were harvested and cultured in triplicate for CFU-C assay as well as FACS analysis. BM homing was calculated as previously reported (Boggs, 1984).

The remainder of the experimental procedures are described in the Supplemental Experimental Procedures section.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.031.

AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

This study was supported by the Heimlich Institute of Cincinnati, the Department of Defense (10580355), the NIH (R01-1HL087159 and HL087159S1), and funds from Hoxworth Blood Center and Cincinnati Children’s Hospital Medical Center (to J.A.C.); R01-CA132847 and R01-CA170225 (to J.M.); R01-CA134530 (to M.T.D.-M.), and R01-AR041255, R01-AR055913, and R01-AR056678 (to R.C.). K.H.C is a National Blood Foundation research fellowship awardee. A.S. was an awardee of The International Lady Tata Memorial Trust, London and acknowledges funding from CSIR, DST-SEERB (SB/SO/HS-053/2013), and Ramalingaswami Fellowship (BT/RRL/RE-ENTRY/06/2010), Department of Biotechnology, India. We thank Cincinnati Children’s Research Foundation Mouse, Flow Cytometry, and Immunobiology Cores for services, supported by the Center of Excellence in Molecular Hematology (P30 DK090971). We also thank Yi Zheng and Hartmut Geiger for critical reading of the manuscript and Ms. Margaret O’Leary for editorial revision.

Received: May 23, 2014
Revised: October 21, 2014
Accepted: November 19, 2014
Published: December 18, 2014

REFERENCES


