Lrp5 Is Not Required for the Proliferative Response of Osteoblasts to Strain but Regulates Proliferation and Apoptosis in a Cell Autonomous Manner

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Abstract

Although Lrp5 is known to be an important contributor to the mechanisms regulating bone mass, its precise role remains unclear. The aim of this study was to establish whether mutations in Lrp5 are associated with differences in the growth and/or apoptosis of osteoblast-like cells and their proliferative response to mechanical strain in vitro. Primary osteoblast-like cells were derived from cortical bone of adult mice lacking functional Lrp5 (Lrp5−/−), those heterozygous for the human G171V High Bone Mass (HBM) mutation (LRP5G171V) and their WT littermates (WTlpr5, WTHBM). Osteoblast proliferation over time was significantly higher in cultures of cells from LRP5G171V mice compared to their WTHBM littermates, and lower in Lrp5−/− cells. Cells from female LRP5G171V mice grew more rapidly than those from males, whereas cells from female Lrp5−/− mice grew more slowly than those from males. Apoptosis induced by serum withdrawal was significantly higher in cultures from Lrp5−/− mice than in those from WTHBM or LRP5G171V mice. Exposure to a single short period of dynamic mechanical strain was associated with a significant increase in cell number but this response was unaffected by genotype which also did not change the ‘threshold’ at which cells responded to strain. In conclusion, the data presented here suggest that Lrp5 loss and gain of function mutations result in cell-autonomous alterations in osteoblast proliferation and apoptosis but do not alter the proliferative response of osteoblasts to mechanical strain in vitro.

Introduction

Normal bone homeostasis is achieved by balancing the number and activity of bone forming osteoblasts and bone resorbing osteoclasts. In the adult skeleton the mechanical strain engendered within the bone tissue by the activities of normal life acts as a stimulus to regulate this osteoblast/osteoclast balance, thereby controlling bone mass and architecture such that the skeleton is of sufficient strength to withstand the loads placed upon it without damage. There is compelling evidence that the co-ordinated activities of many different signalling pathways function to control osteoblast number and activity both basally and in response to mechanical strain [1]. One such pathway is the Wnt pathway. Engagement of the Wnt ligand with the receptor complex comprising of Frizzled and Lrp5/6 stimulates activation of the canonical (involving β-catenin activation) and planar cell polarity pathways. Indeed Wnt signalling has been implicated in the regulation of mesenchymal precursor commitment to the osteoblast lineage [2,3], osteoblast proliferation [4,5], terminal differentiation [6] and apoptosis [7–9]. Humans with an inactivating mutation in the Lrp5 Wnt co-receptor gene have reduced bone mass [6,10,11], whilst individuals with an activating mutation (the G171V mutation) have correspondingly higher than normal bone mass [12–14]. Experimental models such as Lrp5 knockout mice [4] or mice expressing human LRP5 transgene containing the G171V activating mutation (LRP5G171V) [7] generally recapitulate the situation in humans and have low and high bone mass respectively.

Sawakami et al., (2006) provided in vivo evidence that the Wnt pathway may play a role in mediating bone’s adaptive response to loading, by demonstrating that mice lacking functional LRP5 have an impaired cortical bone response to ulna loading [15]. In a recent study that analysed multiple bone responses to graded strains we also demonstrated that absence of Lrp5 activity due to the Lrp5−/− mutation reduces the osteogenic effects of loading in male (but not female) mice, whilst the presence of the LRP5G171V mutated gene was associated with increased mechano-responsive-ness [16]. However, this study supported only a limited gender-related role for LRP5 function in mediating bone’s adaptive response to mechanical loading in vivo.

Given that Lrp5 status in vivo impacts basal and mechanically influenced bone mass, we sought to investigate in vitro whether primary long-bone-derived osteoblast like cells derived from both the Lrp5−/− or LRP5G171V mice displayed cell autonomous differences in their basal growth and apoptosis rates that could explain these physiological phenotypes. We also measured

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proliferation in these cells following exposure to mechanical strain in order to determine whether Lrp5 functionality regulated the magnitude of the strain-related response and/or altered the ‘threshold’ at which a proliferative response to strain was engendered.

**Materials and Methods**

**Ethics statement**

The genetic background of LRP5<sup>G171V</sup> and Lrp5<sup>−/−</sup> is the mouse strain C57BL/6 (Charles River Laboratories, Margate, Kent, U.K.). LRP5<sup>G171V</sup> transgenic and Lrp5<sup>−/−</sup> mice were a gift from Babij et al., (2003) and Kato et al., (2002) respectively [4,7]. Mice from both colonies were housed up to 5 per cage in polypropylene cages with wood chip and paper bedding and provided standard mouse chow and water ad libitum. Weaners up to 8 weeks of age were fed a standard rodent maintenance diet (Special Diet Services, South Witham, UK). All of the procedures conducted in the facility were carried out in accordance with the UK Animals Act (Scientific Procedures) 1986 under a UK Government Home Office project license number PIL70/6350, reviewed and approved by the Royal Veterinary College Local Ethical Review Committee (London, UK).

**Cell extraction**

At 19 weeks of age, LRP5<sup>G171V</sup> and Lrp5<sup>−/−</sup> mice and their WT littermates (WT<sub>LRP5</sub> and WT<sub>Lrp5</sub>) were euthanized by means of cervical dislocation. Primary osteoblast-like cells were isolated from femur, radii, ulnae and humerus as described previously [17]. The osteoblast-like cells were maintained in Dulbecco’s minimal essential medium (DMEM) without phenol-red, 2 mM L-glutamine (Life Technologies Ltd.), 100 U/ml penicillin (Life Technologies Ltd.), and 100 μg/ml streptomycin (Invitrogen Ltd., Paisley, Scotland, UK (Life Technologies Ltd.) supplemented with 10% heat-inactivated foetal calf serum (FCS) (LabTech International, East Sussex, UK) and were incubated together in quadriPERM 4-well plates at 37°C in a humidified 5% CO2 incubator. Only first passage cells (P1) were used.

**Cell proliferation rates**

Primary osteoblast-like cells isolated from female and male LRP5<sup>G171V</sup> and Lrp5<sup>−/−</sup> mice and their WT littermates were seeded onto custom-made sterile, tissue culture-treated plastic strips (Nunc, Dassel, Germany) at a density of 100,000 cells/strip (3 mice of each gender and genotype were used and experiments were repeated three times). Four strips were used per condition and were incubated together in quadriPERM 4-well plates (Greiner Bio-One, Stonehouse, UK) maintained in DMEM containing 10% FCS for 5 days. Mechanical strain was applied using a loading jig that applies four-point bending to each strip with minimal fluid perturbation as described previously [17]. Cells were subjected to a single period of 600 cycles of four-point-bending at a frequency of 1 Hz. This generated a peak strain of 2500, 2900 or 3400 με. Following strain treatment the strips and media were replaced into the 4-well plates and incubated for 48 hours. Control strips were placed in wells of the loading apparatus and subjected to similar perturbation of surrounding media as experienced by the strained strips. However, these strips were not subjected to bending and thus the cells that were plated onto them were not subjected to strain. Cells were stained with propidium iodide (INCYTO, Seoul, South Korea) and counted using a Microchip Type Automatic Cell Counter according to the manufacturer’s instructions (INCYTO, Seoul, South Korea).

**Statistical Analysis**

Statistical significance was determined by a 2-tailed unpaired Student’s t-test or one way analysis of variance (ANOVA) followed by the post hoc (Bonferroni) multiple comparisons between treatment groups using SPSS statistics package version 16 for Windows (SPSS, Chicago, IL, USA). The mixed model analysis was performed using SAS system v9.0 (SAS Institute, Cary, NC, USA). Cell doubling time was calculated using GraphPad Prism v5.0 software for Windows (GraphPad Software Inc., San Diego, CA) by nonlinear regression (exponential growth equation) analysis. Least significant difference was determined and p<0.05 considered statistically significant. Data displayed as mean ± SEM.

**Results**

**Osteoblast proliferation over time in vitro**

Primary osteoblast-like cells extracted from the long bones of female and male LRP5<sup>G171V</sup> and Lrp5<sup>−/−</sup> mice and their WT
littermates were cultured for 2, 4, 6 or 8 days (Fig. 1). There was a main effect of genotype (p<0.001) and time in culture on cell number (p<0.001). The post-hoc analysis showed that cells from LRP5G171V mice proliferated faster than cells from WT littermates (p<0.001), whereas cells from Lp5−/− mice proliferated more slowly than cells from WT Lrp5 littermates. A gender and genotype interaction was detected (p<0.001) together with interaction between gender, genotype and time (p<0.001). The post-hoc analysis also showed that cells from female LRP5G171V mice grew faster than cells from male LRP5G171V, male and female WT and male and female Lrp5−/− mice (p<0.001). Furthermore, female Lp5−/− cells proliferated more slowly compared to male Lp5−/− cells (p<0.001). Doubling time calculated from the regression analysis of each genotype is shown in table 1.

TUNEL staining of cells exposed to serum depletion

Having observed that cells from different genotypes of mice proliferated at different rates, we sought to establish whether this reflected a difference in the rate of apoptosis. Significantly higher levels of TUNEL stained cells were observed in cultures from Lrp5−/− mice compared to cultures from WT littermates (p<0.001) (Fig. 2). In contrast, the percentage of TUNEL stained cells from LRP5G171V mice was significantly lower compared to Lp5−/− and WT cells (p<0.001). There was an effect of gender, genotype and serum concentration on percentage of TUNEL stained cells (p<0.001) and an interaction between genotype and serum concentration was also detected (p<0.001). In 0.1 and 2.5% serum the percentage of TUNEL positive osteoblast-like cells was significantly higher in cultures of female and male Lrp5−/− cells compared to cultures of female and male WT and LRP5G171V cells (p<0.001 and p<0.01 respectively). Similar to the situation in 0.1 and 2.5% serum, the percentage of TUNEL positive cells in 10% serum was significantly higher in cultures of female and male Lrp5−/− cells compared to female and male WT and LRP5G171V cells (p<0.05). Gender related differences in the percentage of TUNEL positive cells were also observed within each genotype; in 2.5% and 10% serum the percentage of TUNEL positive cells was higher in cultures of male WT HBM cells compared to cultures of female WT HBM cells (p<0.05) and no gender differences in TUNEL positive cells was observed in 0.1% serum. In contrast, in 2.5% serum the percentage of TUNEL positive cells was higher in cultures of female Lp5−/− cells compared to cultures of male Lp5−/− cells (p<0.01) and no gender differences in 0.1% and 10% was observed.

Table 1. Cell population doubling time of primary osteoblast-like cells derived from female and male Lrp5−/−, LRP5G171V and their WT littermates.

| Genotype       | Double time (days) |
|----------------|--------------------|
| Female WT      | 3.42               |
| Female Lrp5    | 3.26               |
| Male WT        | 3.26               |
| Male Lrp5      | 3.36               |
| Female LRP5    | 2.52               |
| Male LRP5      | 2.96               |
| Female Lrp5−/− | 4.34               |
| Male Lrp5−/−   | 4.14               |

Doubling time in days between 2 and 8 days of culture of primary osteoblast-like cells isolated from female and male Lrp5−/−, LRP5G171V and their WT littermates. Cell doubling time were calculated using GraphPad Prism v5.0 software for Windows (GraphPad Software Inc., San Diego, CA) by nonlinear regression (exponential growth equation) analysis. doi:10.1371/journal.pone.0035726.t001

Figure 1. Proliferation of primary osteoblast-like cells derived from female and male LRP5G171V and Lrp5−/− mice and their WT littermates. Osteoblast-like cells were cultured over 8 days and were fixed in absolute ice-cold MeOH on day 2, 4, 6 and 8. Cell’s nuclei were stained using propidium iodide and counted using Microchip Type Automatic Cell Counter machine. Results are the mean ± SEM of three independent experiments. N = 4. Groups with the same letter are not significantly different. b vs. a = P<0.001. a + b vs. c = P<0.001.

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Osteoblast proliferation in response to mechanical strain in vitro

The proliferative response of osteoblast-like cells cultured from each gender and genotype of mice to different magnitudes of mechanical strain is shown in figure 3. All groups showed no response at 2,500 με and 2,900 με however, for each gender and genotype (p<0.001) a significant increase in cell number was detected at 3,400 με (Figs. 3A, B and C). We also calculated the percentage increase in cell number between the control (static) and strained groups and found no significant differences between female and male or LRP5<sup>G171V</sup>, Lrp5<sup>2/2</sup> and WT cells (Fig. 3D).

Mixed model analysis showed a main effect of gender (p<0.001), genotype (p<0.001) and strain (p<0.001) on cell number. A genotype and strain interaction was detected (p<0.006) and an interaction between gender, genotype and strain (p<0.001). As described previously, the number of cells in static control cultures of female and male LRP5<sup>G171V</sup>, Lrp5<sup>2/2</sup> and WT cells (Fig. 3D).

Discussion

LRP5 plays a role in osteoblast proliferation

The key finding from this study was that absence of Lrp5 function in osteoblastic cells derived from the cortical bone of adult mice was associated with decreased proliferation as evidenced by an increase in cell population doubling time compared with WT<sub>WTHBM</sub> cells in vitro. Conversely, presence of the LRP5<sup>G171V</sup> mutation in osteoblastic cells was associated with increased proliferation compared with Lrp5<sup>2/2</sup> and WT cells. Cell
population doubling times were comparable in the cultures of female and male WT1/2 and WT_Lrp5 cells, suggesting that WT cells from both backgrounds have a similar proliferation rate. These findings provide evidence that the presence of functional LRP5 protein plays an important role in the regulation of osteoblast proliferation, which is in broad agreement with previous studies [4,6].

These results are not consistent with those of Yadav et al., (2009) who observed no significant differences between the proliferation of Lbp5−/− and WT_Lbp5 osteoblast-like cells [18]. However, differences between our two studies could potentially explain these apparently contrasting observations. The first relates to the site of origin of the cells; the study described here used osteoblast-like cells derived from long bones, whereas Yadav et al. (2009) used calvarial derived osteoblast-like cells. Several years ago our group demonstrated that osteoblastic cells from long bones and calvariae respond differently to a strain-related stimulus [19]. More recently another study confirmed that primary osteoblast-like cells derived from calvariae or long bones are both phenotypically different in vitro and also are significantly different at the level of gene expression [20]. Second, the age of animals was very different in the two studies; Yadav et al. (2009) derived osteoblast-like cells from newborn mice whereas cells from adult (19 week old) mice were used in the present study. Because pre-osteoblasts and osteoblasts from young humans and animals proliferate more rapidly than cells from older animals [21–29], this potentially could ‘mask’ the effect on proliferation of the loss of Lbp5. It must also be considered that the long-term loss of Lbp5 (from birth to 19 weeks of age) has different effects to loss of the gene during development only. Kato et al., (2002) demonstrated that bone formation was normal in Lbp5−/− mice at 17.5 days post coitum and at birth there was only a subtle delay in osteogenesis. However, the ossification defect in these mice became more pronounced with age and reflected a defect in osteoblast proliferation. Finally, different promoters were used in the two studies. The Lrp5G171V mice used for the present study were generated by Babij et al. (2003) using the 3.6 kb fragment of the collagen type 1 promoter to drive the expression of the transgene in pre-osteoblasts and osteoblasts, with minimal to no expression in other cell types [30,31]. In contrast, Yadav et al., (2009) used the 2.3 kb collagen type 1 promoter to replace one copy of the endogenous murine Lbp5 gene with one copy of Lbp5 carrying the high bone mass G171V mutation. This generated mice with one WT and one G171V allele, rather than two WT and one G171V allele as used here. The 2.3 kb promoter driving Cre expression is activated later in the osteoblast differentiation pathway than the 3.6 kb promoter, and is thus active in more mature osteoblasts [30].

We have also found that the effect of Lbp5 mutations on osteoblast proliferation was more pronounced in cells from female mice, such that Lbp5 gain of function stimulated whilst Lbp5 loss of function impaired proliferation. We have previously shown that ERα is required for β-catenin function in response to strain and it is now apparent that this is mediated, at least in part, by the nongenomic signalling effects of ER involved with IGFIR’s action [32,33]. This suggests that there are gender differences in LRP5-β-catenin signalling in osteoblasts, with females being more affected by changes in its activity because of potential interactions with the E2/ERα IGF-I signalling pathways. Interestingly, it has been shown that bone marrow stromal cells isolated from young women express higher levels of the ER and the ERR target gene Wnt11. Conversely, male BMSCs express higher levels of Wnt 16, which has two isoforms associated with either senescence or proliferation [34,35].

LRP5 is involved in osteoblast apoptosis

Having demonstrated differences in proliferation of cells from different genotypes, we studied the effect of LRP5 mutations on in vitro and in vivo which showed that loss of LRP5 function increased apoptosis in primary osteoblast-like cells from Lbp5−/− mice. This result suggest that the low bone mass phenotype observed in vivo may reflect, at least in part, high levels of apoptosis in the bone cells of these mice. This finding is in agreement with a previous in vitro study in which apoptosis in calvarial-derived osteoblast-like cells from Lbp5−/− mice was shown to be higher compared to WT cells [9]. However, our data is not in agreement with a previous in vivo study by Kato et al., (2002), in which no difference in osteoblast apoptosis rates were observed in calvarial sections from Lbp5−/− mice. The LRP5 G171V mutation seems to provide some protection against apoptosis induced by serum depletion however, the effect of this mutation on osteoblast apoptosis does not appear to be as significant as the loss of LRP5 function. Notwithstanding, our findings support the idea that LRP5 is a critical component in the regulation of bone cell apoptosis [7–9,36,37].

LRP5 is not required for the proliferative response of osteoblasts to strain

One of the effects of mechanical strain is to stimulate proliferation of cells that are, or will become, osteoblasts [38–47]. In the studies reported here neither absence of LRP5 function nor the presence of the LRP5G171V mutation altered the proliferative response of cortical derived primary osteoblast-like cells to mechanical strain in vitro. These data are in agreement with two recent in vitro studies which suggested that the strain-induced activation of β-catenin does not require LRP5 [33,48,49], although a previous in vivo study had shown that the loading response was abolished in mice lacking LRP5 [15].

Neither did the Lbp5−/− nor the LRP5G171V mutation alter the ‘minimum effective strain’ at which strain engendered a proliferative response, thus our findings are not consistent with in vivo evidence that a lower strain threshold is sufficient to induce cortical bone formation in LRP5G171V mice [30,51]. We have also recently demonstrated that the increased load-induced osteogenesis in the cortical and cancellous bone of mice with the LRP5G171V mutation is more pronounced in females than males [16]. One possible explanation for the apparent differences between our in vitro data and in vivo findings is that LRP5 mutations may alter the strain responsiveness in osteocytes rather than osteoblasts. For example, differences in responses to fluid flow or shear stress have been reported in osteocytes versus osteoblasts [49,52,53]. Our in vitro model includes osteoblasts with little or no osteocytic component and so does not replicate the complex context of cortical bone.

In conclusion, we have provided data to demonstrate that in cortical bone-derived primary osteoblast-like cells from adult mice, LRP5 is an integral component of the signalling pathways that regulate cell proliferation and apoptosis. However, it is not required for the proliferative response of these cells to mechanical strain. The intrinsically higher rate of proliferation and reduced apoptosis observed in the LRP5G171V osteoblastic cells may result in an increased amount of new bone being formed which would partially explain the high bone mass seen in individuals with this mutation.

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Author Contributions
Conceived and designed the experiments: BJ AS GZ LKS LEL JSP. Performed the experiments: BJ RFLS. Analyzed the data: BJ AS GZ LEL.

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