Title: Mechanical Signals Promote Osteogenic Fate Through a Primary Cilia Mediated Mechanism

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Short Title:

Non-standard Abbreviations

Kif3a	Kinesin family member 3A
GFP	Green Fluorescent Protein
Cre-ER	tamoxifen inducible Cre-recombinase
Gy	Gray
B.Pm	Bone Perimeter (B.Pm),
sL.Pm	Single Label Perimeter
dL.Pm	Double Label Perimeter
dL.Ar	Double Label Area
MS/BS	Mineralizing surface
MAR	Mineral Apposition Rate
BFR	Bone Formation Rate
rMS/BS	Relative Mineralizing surface
rMAR	Relative Mineral Apposition Rate
rBFR	Relative Bone Formation Rate
PDGFα	Platelet Derived Growth Factor alpha

Abstract: It has long been suspected, but never directly shown, that bone formed to accommodate an increase in mechanical loading is due to the creation of osteoblasts from skeletal stem cells. Indeed, biophysical stimuli potently regulate osteogenic lineage commitment *in vitro*. In this study, we transplanted bone marrow cells expressing GFP to enable lineage tracing and subjected mice to a biophysical stimulus to elicit a bone forming response. We detected cells derived from transplanted progenitors embedded within the bone matrix near active bone forming surfaces in response to loading, demonstrating for the first time, that mechanical signals enhance the homing and attachment of bone marrow cells to bone surfaces and osteogenic lineage commitment of these cells in vivo. Furthermore, we used an inducible Cre/lox recombination system to delete Kif3A, a gene important for primary cilia formation, at will in transplanted cells and their progeny, regardless of which tissue they may have incorporated into. Disruption of the mechanosensing organelle, the primary cilium, in a progenitor population significantly decreased the amount of bone formed in response to mechanical stimulation. Collectively, our study directly demonstrates, utilizing a novel experimental stem cell mechanobiology model, that mechanical signals enhance osteogenic lineage commitment *in vivo* and that the primary cilium contributes to this process.

Keywords:

Mesenchymal Stem Cell

Mechanical Loading

Primary Cilium

Bone

Homing

Introduction:

Bones are well known to be sensitive to mechanical loading. While a decrease in mechanical loading may result in bone loss, an increase in loading promotes bone formation. Given the finite lifespan and non-proliferative state of the bone forming osteoblast (1), continued bone formation in response to any stimulus must require the recruitment of osteoprogenitors cells (2). In an attempt to demonstrate this by tracing the osteogenic lineage of marrow derived cells, several studies have used chimeric animals, whereby labeled marrow is transplanted into a lethally irradiated host. Such models have demonstrated that progenitor cells home to the marrow cavity and contribute to bone maintenance and fracture repair (3-5). Although it is generally expected that the process of loading-induced bone formation involves the recruitment of progenitors (6, 7), surprisingly, the cellular origin of bone forming osteoblasts in this process remains unclear. Specifically, verification through lineage tracing has never been performed in response to mechanical loading. While *in vitro* studies have demonstrated that biophysical stimulation enhances osteogenic lineage commitment for stem cells (8-11), it is also possible that loading may instead activate existing dormant osteoblasts and/or bone lining cells *in vivo* (12). Identifying the source of bone forming cells in response to physical stimuli could inform development of effective therapies for enhancing bone formation in disease.

A major challenge in mechanobiology currently is deciphering how cells sense a biophysical signal and transduce that signal into a biochemical response. One proposed mechanism by which stem cells may sense their mechanical environment is through the primary cilium. Primary cilia are antenna-like organelles that protrude from the cell body, and serve as microdomains, concentrating and enhancing the kinetics of signaling molecules (13). Recent *in vitro* studies have implicated the primary cilium in mesenchymal stem cells (MSCs) as both a chemosensor (14, 15), as well as a mechanosensor (10) important for osteogenic differentiation, with ~25-90% of MSCs possessing a cilium (10, 16). Although identification of stem cell primary cilia *in vivo* has proved challenging, recent studies have demonstrated that only 1% of marrow cells possess a primary cilium in ovine bone. However, given that only 0.01% of marrow cells constitute the MSC population (17) of marrow and that the majority of cells derived from the hematopoietic system do not possess a cilium, it is hypothesized that

MSCs *in vivo* demonstrate similar high cilia incidence to that as demonstrated *in vitro*. Importantly, bone formation in response to mechanical loading has been demonstrated to be mediated by bone cell primary cilia *in vivo* (18). However, the role of primary cilia in progenitor cells for loading induced bone formation *in vivo* is unknown.

Given the importance of understanding the cellular mechanisms behind bone formation for the development of treatments for bone loss diseases, this study aimed, using a novel bone marrow transplant model, to elucidate the role of the marrow progenitor cell in loading induced-bone formation and further elucidate the role of the progenitor primary cilium in this process. In this study we have directly demonstrated, for the first time, marrow progenitor cell participation in mechanical loading driven bone anabolic responses *in vivo*. Furthermore we have demonstrated that the progenitor cell primary cilium mediates this loading induced response, highlighting this organelle as a potential therapeutic target to activate the progenitor population.

Materials and Methods:

Mice. All animal protocols were approved by the Institutional Animal Care and Use Committee at Columbia University. All mice, except Kif3a$^{fl/fl}$ mice, were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in the animal facility at Columbia University. Kif3a$^{fl/fl}$ mice were recovered from a cryo-archive at the UC Davis Mutant Mice Regional Resource Center. Kinesin family member 3A (Kif3a) is an intraflagellar transport protein that is important for primary cilia formation, maintenance and function (22, 23), and its global deletion results in embryonic lethality (20, 24). Kif3a$^{fl/fl}$ mice were bred with a GFP expressing animal to enabled us to track bone marrow cells as well as their progeny resulting in a Kif3a$^{fl/fl}$;GFP control animal and further crossed with a mouse that expressed an endogenous tamoxifen-inducible Cre allele driven by the ROSA26 locus (19) to obtain a Kif3a$^{fl/fl}$;GFP;Cre-ER^{T2} experimental animal. As global deletion of Kif3a results in embryonic lethality, to study the effect of deleting Kif3a in stem cells, we performed bone marrow transplants to repopulate only the bone marrow with cells in which deletion of Kif3a could be performed at will. Wild type mice were treated with a lethal dose of whole body irradiation to deplete quickly regenerating cells,

and bone marrow cells isolated from donors Kif3afl/fl;Cre-ER^{T2};GFP were transplanted to create experimental animals. Cells from Kif3a$^{fl/fl}$;GFP mice were also transplanted to create control animals.

Bone Marrow Transplant. 11-week old wild type male C57BL/6 host mice were subjected to whole body irradiation using a Gammacell 40 cesium irradiator (Atomic energy, Ontario, Canada). Host mice received two lethal irradiations of 6 Gy separated by 4 hours (19). Immediately after the second dose, bone marrow cells were transplanted via tail vein injection. Fresh bone marrow cells were isolated from six to eight week old control (Kif3a$^{fl/fl}$;GFP) or experimental (Kif3a$^{fl/fl}$;Cre-ER^{T2};GFP) male donor mice. Donor mice were sacrificed, and tibias and femurs were collected. Bone marrow cells were isolated by flushing the bone marrow cavity with alpha modified minimal essential medium (Life Technologies, Grand Island, NY) using 27.5 G syringes. Cells were passed through a 70 um cell strainer, centrifuged and resuspended in PBS. 20 million nucleated cells were injected for transplantation. Animals were monitored daily for 2 weeks after transplant.

Fluorescence activated cell sorting. To verify that donor cells successfully engrafted and reconstituted the hematopoietic system, blood cells were isolated and treated with ACK lysis buffer (Life Technologies, USA) for 30 seconds to lyse non-nucleated cells. Cells were detected for the presence of GFP using a flow cytometer (FACSCanto II, BD, Franklin Lakes, NJ, USA). Graphs were analyzed using FlowJo software. Data were analyzed using the same gate value for which >99% of cells obtained from a GFP transgenic animals were analyzed as positive for GFP, as well as 100% negative for cells obtained from a wild type animal.

Bone marrow culture. To verify that donor cells successfully engrafted and reconstituted the bone marrow stromal cell population, cells were isolated as described above, and seeded in tissue culture polystyrene dishes. Plastic adherence has been proposed by the International Society for Cellular Therapy to be a criterion for defining BMSCs (27), and we used this quality to separate BMSCs from the heterogeneous bone marrow population. Cells were cultured using low glucose Dulbecco's Modified Eagle Medium (Life Technologies,

USA) supplemented with 10% fetal bovine serum (Hyclone, Pittsburgh, PA, USA) and 1% penicillin and streptomycin (Life Technologies, USA). Media was changed daily for the first 5 days to remove non-adherent cells. Media was changed once every 2 or 3 days following until 2 weeks of total culture time.

DNA isolation and PCR. Freshly isolated bone marrow cells were pelleted and DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). PCR was performed to detect Kif3a floxed, wild type, and deleted targets as previously described (20).

Mechanical loading. Prior to loading, 15 week old animals were injected daily for 5 days with tamoxifen (25 mg/kg body weight, Sigma Chemical Co., St. Louis, MO, USA) to induce Cre recombination. At 16 weeks, skeletally mature animals were subjected to mechanical loading of the ulnae using an electromagnetic loading system (EnduraTEC, Bose, Eden Prairie, MN, USA) (21). While under isoflurane anesthesia, the right forelimb was axially loaded at 3 N at 2 Hz for 120 cycles per day for 3 consecutive days. Left forelimbs remained nonloaded to serve as internal controls. Animals received subcutaneous injections of fluorochrome calcium binding labels Calcein (30 mg/kg body weight, Sigma) 4 days after the first day of loading and Alizarin Red S (70 mg/kg body weight, Sigma) 8 days after the first day of loading.

Immunohistochemistry. Animals were euthanized 14 days after the first day of loading. Both ulnae were carefully dissected out and fixed in 10% formalin solution (Sigma, USA). Samples were decalcified in RDO, dehydrated through ethanol and xylene, embedded in paraffin, and transverse sections at the midshaft of the ulnae were cut at 5 um. Sections were deparaffinized, rehydrated, treated for antigen retrieval, blocked in goat serum, and then incubated with primary antibodies against GFP (1:500, Life Technologies, A11122) overnight. Sections were then incubated with secondary antibodies (Vectastain ABC kit (Rabbit IgG), Vector Laboratories, PK-4001, USA) for 30 min, followed by ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min.

Finally, sections were incubated in diaminobenzidine (DAKO, Carpinteria, CA, USA) substrate solution until developed, and counterstained in hematoxylin.

Dynamic histomorphometry. Both ulnae for each mouse were isolated and stored in 70% ethanol. Ulnae were dehydrated through ethanol and xylene, infiltrated with methyl methacrylate and dibutyl phthalate, and embedded in methyl methacrylate and dibutyl phthalate in the presence of benzoyl peroxide. Transverse sections at the midshaft of the ulnae were cut using a diamond saw (IsoMet Low Speed Saw, Buehler, Lake Bluff, IL, USA) and imaged on a laser scanning confocal microscope (Leica TCS SP5, Leica Microsystems Inc., Buffalo Grove, IL,USA). Using ImageJ software, the following measures were taken from the periosteal surface: bone perimeter (B.Pm), single label perimeter (sL.Pm), double label perimeter (dL.Pm), and double label area (dL.Ar). Bone formation parameters were calculated from these measures. Mineralizing surface (MS/BS = [1/2 sL.Pm + dL.Pm]/B.Pm x 100; %), mineral apposition rate (MAR = dL.Ar/dL.Pm/4 days; μm per day) and bone formation rate (BFR/BS = MAR x MS/BS x 3.65; $\mu m^3/\mu m^2$ per day). To determine the portion of bone formation rate due to mechanical loading, relative (r) measurements of rMS/BS, rMAR and rBFR/BS were calculated by subtracting the value for the nonloaded forelimb from the loaded forelimb for individual animals.

Statistical analysis. Data are presented as mean +/- SEM. Student's *t*-test was used to calculate *p* values using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Paired two-tailed *t*-tests were used for comparing differences between loaded to nonloaded values. Unpaired two-tailed *t*-tests with unequal variances were used for comparing differences in relative bone formation values between animals with deletion of Kif3a in bone marrow cells and controls. For all tests, $\alpha = 0.05$.

Results:

To establish the lineage of bone forming osteoblasts in mechanically induced bone formation and determine if primary cilia are involved, we generated chimeric mice whereby marrow from experimental donor animals were transplanted into lethally irradiated wild type host animals. To verify the generation of chimeric animals, we confirmed that depletion of host bone marrow cells and successful engraftment of donor cells had occurred. We visually verified that in animals receiving transplants, most bone marrow cells expressed GFP, indicating donor origin (Fig. 1A). We also utilized flow cytometry to observe 95% of nucleated cells derived from whole blood to be GFP positive (Fig. 1B), demonstrating that donor cells had reconstituted the hematopoietic system. While this confirmed successful engraftment of hematopoietic stem cells, we also had to determine if bone marrow stromal cells (BMSCs) had successfully engrafted. We cultured cells isolated from bone marrow on plastic as proposed by the International Society for Cellular Therapy, and were able to visually detect GFP in a majority of plastic-adherent cells (Fig. 1C), indicating that BMSCs were primarily donor derived.

To confirm that effective deletion of Kif3a in bone marrow cells could be achieved, we isolated DNA from bone marrow cells of tamoxifen treated chimeric animals and used PCR to amplify Kif3a targets. In experimental animals, a majority of amplified targets were for the deleted Kif3a allele (Fig. 1D), indicating effective recombination. In control animals, the deleted Kif3a allele was not detected, and the majority of amplified targets were for the Kif3a floxed allele. Furthermore, PCR bands for the wild type version of Kif3a were very faint for both control and experimental chimeric animals, indicating that the vast majority of bone marrow cells were donor derived rather than host derived.

To investigate bone formation in response to mechanical loading, we loaded the forelimb of skeletally mature 16 week old chimeric animals and administered fluorochrome calcium binding labels Calcein and Alizarin Red on Days 5 and 9 respectively to mark regions of new bone formation (Fig. 2A). There was no visual difference in general cage activity between groups post loading.

To determine the cellular origin of bone cells, we probed for GFP expression through immunohistochemistry on cross sections at the midlength of loaded and nonloaded ulnae. We observed that mechanical loading increased the number of GFP positive cells embedded within bone (Figs. 2B, C). Bone marrow derived cells were

detected at both the endocortical and periosteal regions of mechanically loaded bones (Fig. 2C). In the nonloaded contralateral control ulnae, bone marrow derived cells were also detected in the endocortical region, but their presence in the periosteal region was decreased compared to loaded bone (Fig. 2D). This demonstrates that mechanical stimulation promoted the creation of bone forming osteoblasts and osteocytes from progenitors. In experimental animals, cells in which Kif3a was deleted were also detected embedded within bone (Fig. 2E), indicating that cells with disrupted primary cilia retained their inherent abilities to differentiate towards the osteogenic lineage and to form bone.

To determine if mechanical loading stimulated an increase in bone formation rates for both our control and experimental animals, we used dynamic histomorphometry to visualize areas of active bone formation (18). In response to ulnae compressive loading, the %MS significantly increased in both the control (3.52-fold, n=5) and experimental animals (2.93-fold, n=9), the MAR significantly increased in both control (2.52-fold, n=5) and experimental animals (2.68-fold, 2=9) and also the BFR significantly increased in both control (7.89-fold, n=5) and experimental animals (5.55-fold, n=9) when compared to nonloaded ulnae within groups (Figs. 3B-D)

To analyze the effect of primary cilia on loading induced bone formation, we compared relative rates of bone formation between control and experimental animals. We calculated relative (r) rates by subtracting the value of the nonloaded ulna from the loaded ulna. Therefore, the relative rate does not include the baseline bone formation rate and captures the proportion of bone formation that is a response to mechanical loading. This response was attenuated in animals where primary cilia were disrupted in bone marrow derived cells. The percent of the periosteal surface that was activated for bone formation was decreased by nearly half (45%, $p<0.05$) compared to animals with intact primary cilia (Fig. 3E). However, within activated areas, the rate of bone formation was not affected by the absence of primary cilia (Fig. 3F). The total bone formation rate is the product of the previous measures (percent of the activated periosteal surface x formation rate in these areas), and due to the decrease in activated surface area, this was decreased overall (Fig. 3G).

Discussion:

Bone diseases resulting from low bone mass such as osteoporosis are a severe health threat. One avenue to combat these diseases is to encourage an increase in bone formation. While mechanical loading has long been known to be a potent stimulus, the origin of bone forming cells involved in this process and how these cells sense mechanical signals is poorly understood. Progenitor cells differentiate to osteoblasts during development, and have long been thought to be a natural source for new active osteoblasts during loading induced bone formation. In this study, we directly demonstrate that mechanical stimulation promotes osteogenic lineage commitment and migration of progenitor cells *in vivo* leading to enhanced bone formation and furthermore demonstrate that this osteogenic response is mediated in part by the primary cilium in the progenitor population. Our findings therefore highlight not only the osteoprogenitor, but importantly the primary cilium, as novel therapeutic targets for bone loss diseases such as osteoporosis.

Mechanical loading was shown to enhance the number of bone marrow derived cells embedded within bone demonstrating that in response to a physical stimulus, engrafted progenitor cells homed to the bone surface and underwent osteogenic lineage commitment. An important finding is that bone marrow derived cells were detected in both the endocortical and periosteal regions of mechanically loaded bones. Since the endocortical region is in intimate contact with bone marrow, we were not surprised to detect bone marrow derived cells in this region. However, many intriguing possibilities exist for how bone marrow derived cells may have arrived at the periosteal region. It is possible that cells migrated from the bone marrow, were circulating, or were present in the periosteum or muscle prior to mechanical loading. Possible mechanisms for activation of the periosteal bone surface in response to loading include recruitment and subsequent osteogenic differentiation of progenitor cells, or alternatively activation of previously dormant osteoblasts that were already present on the bone surface. Interestingly, as we observed a decrease in loading induced periosteal activation in bone where the cilium was deleted in the progenitor population and not the osteoblast, this would suggest that loading induced periosteal activation is due to the recruitment and differentiation of osteogenic progenitors and not dormant osteoblasts. One potential mechanism for recruitment and differentiation is that osteocytes, previously demonstrated to be important in bone mechanotransduction (22), detect mechanical stimulation, and then

release paracrine signals, which are detected by progenitor cells (23-25). Recent work by our group has demonstrated that soluble factors secreted by mechanically stimulated osteocytes can significantly enhance MSC migration *in vitro* (24). However, an alternative mechanism may be that progenitor cells directly sense these biophysical signals. It is clear from these findings that progenitor cells are essential for loading induced bone formation but whether their contribution is regulated directly by physical stimulus or indirectly through a coordinating cell such as the osteocyte remains unclear.

Mechanical loading induced bone formation was inhibited in animals with defective marrow cell primary cilia demonstrating an important role for the cilium in mediating skeletal adaptation. Interestingly, within an activated area, the rate of bone formation was not affected by the absence of primary cilia (Fig. 3F). Rather, the higher total bone formation we observed in the control group was due to a larger portion of the surface being activated. This suggests that once cells with disrupted primary cilia differentiate to osteoblasts, the absence of primary cilia does not influence osteoblast activity. In contrast, Tummala *et al* demonstrated reduced RUNX2 expression in hMSCs with defective cilia (14). This may indicate that MSC osteogenic differentiation may be delayed but can recover with time. Interestingly, Qiu *et al*, using an osteoblast specific primary cilia deletion model, discovered that mice at 6 weeks were osteopenic yet recovered at 24 weeks (26). Furthermore, Temiyasathit et al. found no decrease in activated area with loading, but osteoblast activity was decreased when primary cilia were disrupted in osteocytes and osteoblasts (18). This suggests that primary cilia in osteocytes and osteoblasts influence how quickly bone is laid down in active areas, while primary cilia in progenitor cells influence the amount of area that is activated, potentially by regulating stem cell homing. Interestingly, the primary cilium has previously been shown to coordinate PDGFα induced directional migration in fibroblasts by acting as a signalling microdomain for this pathway (27). Although a significant inhibition of loading-induced bone formation was demonstrated in animals with defective cilia in marrow derived cells, bone formation still occurred. Bone marrow stromal cells may compensate for defective cilia through activation of other mechanosensory complexes such as at focal adhesions (28). Furthermore, this also raises the possibility that other precursors may exist that contribute to the osteoblast population in these experimental animals. For

example, host progenitors which were not ablated by irradiation may still contribute to this response. Another important consideration for our study is that Kif3a was deleted not only in osteoprogenitors, but also in all bone marrow cells. This raises the possibility that the attenuated bone formation is actually the result of disrupting primary cilia in cells regulating the stem cell niche. The stem cell niche is a dynamic microenvironment that contains the stem cell and supporting cells that influence stem cell fate. Support cells may include hematopoietic stem cells, endothelial cells, osteoblasts, and adipocytes (29), all of which are present in bone marrow.

An intriguing question that remains is whether loading promotes migration of progenitors in the bone marrow to the periosteal surface, or if osteoblasts are derived from progenitors residing in stem cell niches in the periosteum or even muscle. We show here that primary cilia in progenitor cells are crucial in facilitating mechanically induced skeletal adaptation. However, the specific role of progenitor cell primary cilia remains unclear. Primary cilia could potentially be important in proliferation (10), migration from the stem cell niche (27), homing (30), initial asymmetric division (31), or differentiation (10, 14). Our study demonstrating stem cell regulation by mechanical signals and primary cilia as a mechanism for sensing these signals opens exciting avenues for future research. Understanding how stem cell differentiation and activity are regulated is applicable not only for addressing bone diseases, but is also important for developing a broad range of regenerative therapies.

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Any Additional Author notes:

Author Contributions:

J.C.C. designed and carried out experiments, interpreted data and wrote the manuscript. D.A.H. designed and carried out experiments, interpreted data and wrote the manuscript. M.C. and R.B. performed experiments. C.R.J. designed experiments, interpreted data and wrote the manuscript.

Figure Captions:

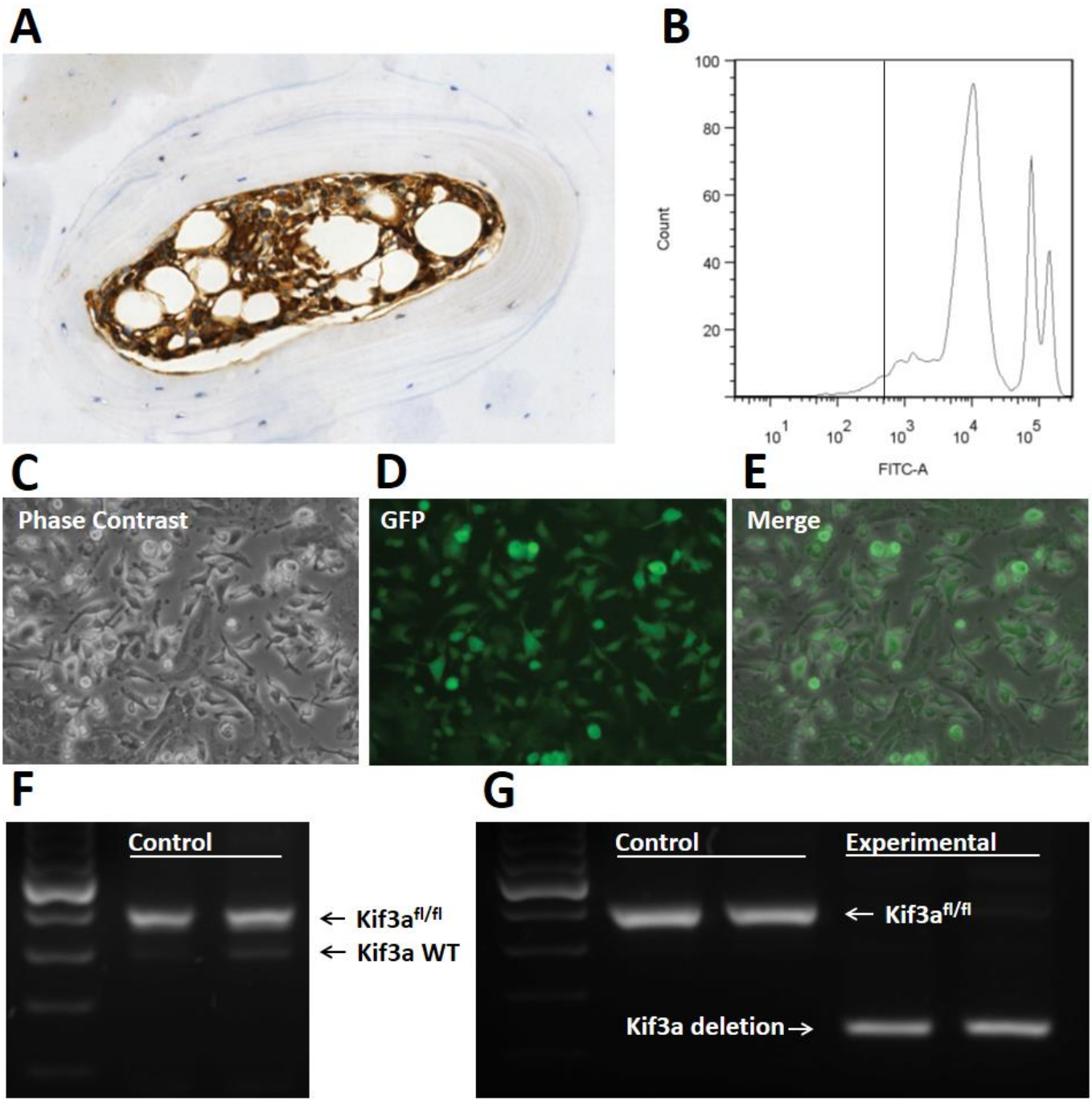

Fig. 1. Generation of chimeric animals. **(A)** Histological analysis of donor engraftment. GFP positive (brown) donor cells were detected in the bone marrow. **(B)** Fluorescence Activated Cell Sorting representative analysis. 95.4±1.2% of nucleated blood cells were positive for GFP. **(C-E)** Cells were isolated from bone marrow, and the plastic-adherent population was analyzed for GFP. The vast majority of adherent cells were positive for GFP **(C)** Phase Contrast, **(D)** GFP, **(E)** Merge. **(F)** DNA was extracted from isolated bone marrow cells. PCR

amplification demonstrates that the floxed allele of Kif3a was amplified in the control animal with very faint amplification of wild-type Kif3a indicating the marrow stromal cell population was repopulated by transplantation. **(G)** PCR amplification of marrow stromal cells isolated from tamoxifen treated control animals demonstrates expression of the floxed allele of Kif3a, while experimental animals expresses the deleted Kif3a target, demonstrating successful deletion of Kif3a in the experimental group.

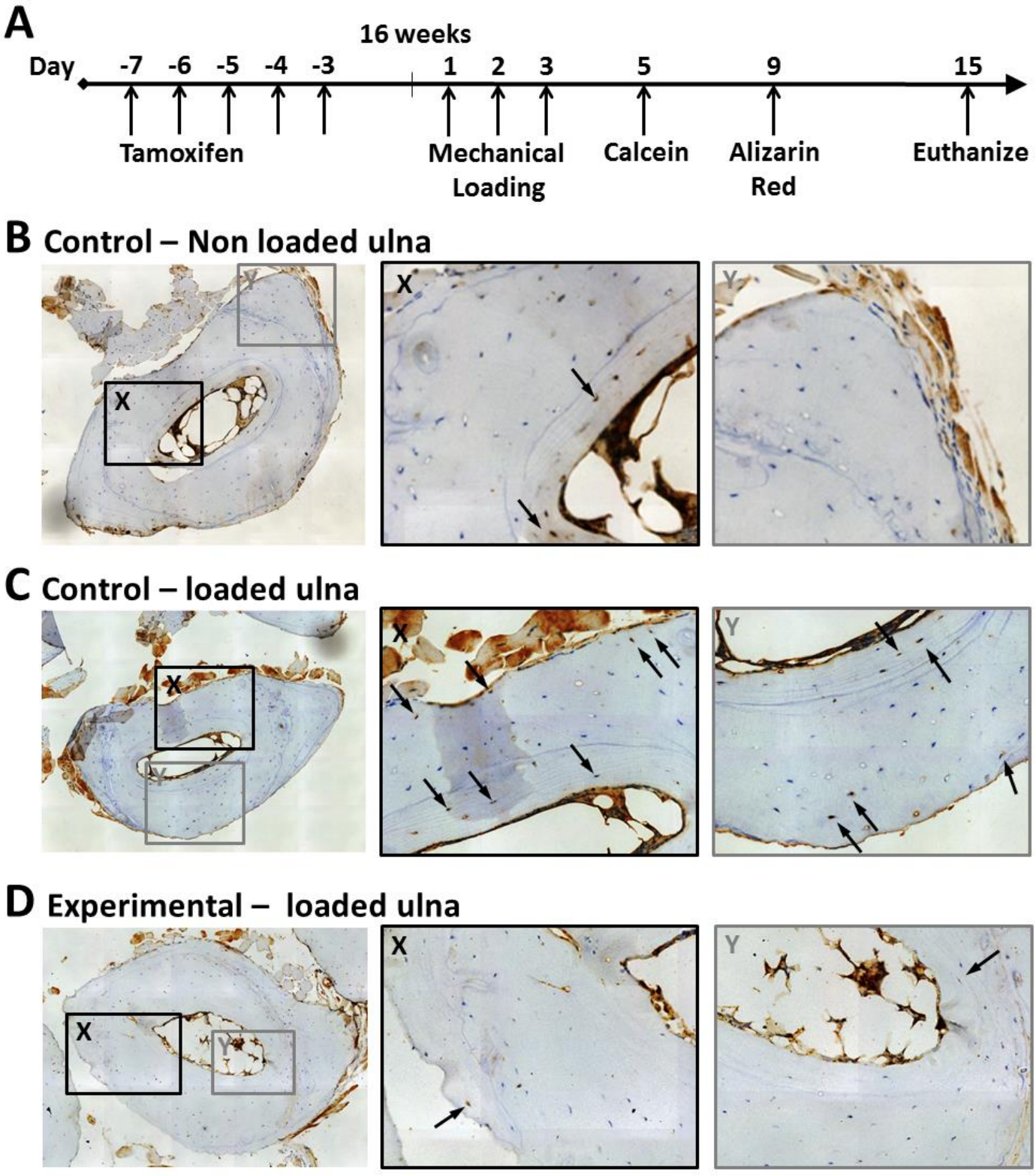

Fig. 2. Mechanical stimulation promotes osteogenic differentiation of bone marrow derived cells. **(A)** Experimental timeline. **(B)** 16 week old control animal, Non loaded control. Bone marrow derived cells were only detected within the endocortical region **(C)** Control animal, after mechanical loading. Bone marrow

derived cells were embedded within the endocortical and periosteal bone. **(D)** Experimental animal after mechanical loading. Bone marrow derived cells were also embedded in endocortical bone, but less were detected in the periosteal region compared to control animals illustrated in **(C)**. Arrows indicate donor bone marrow derived osteocytes.

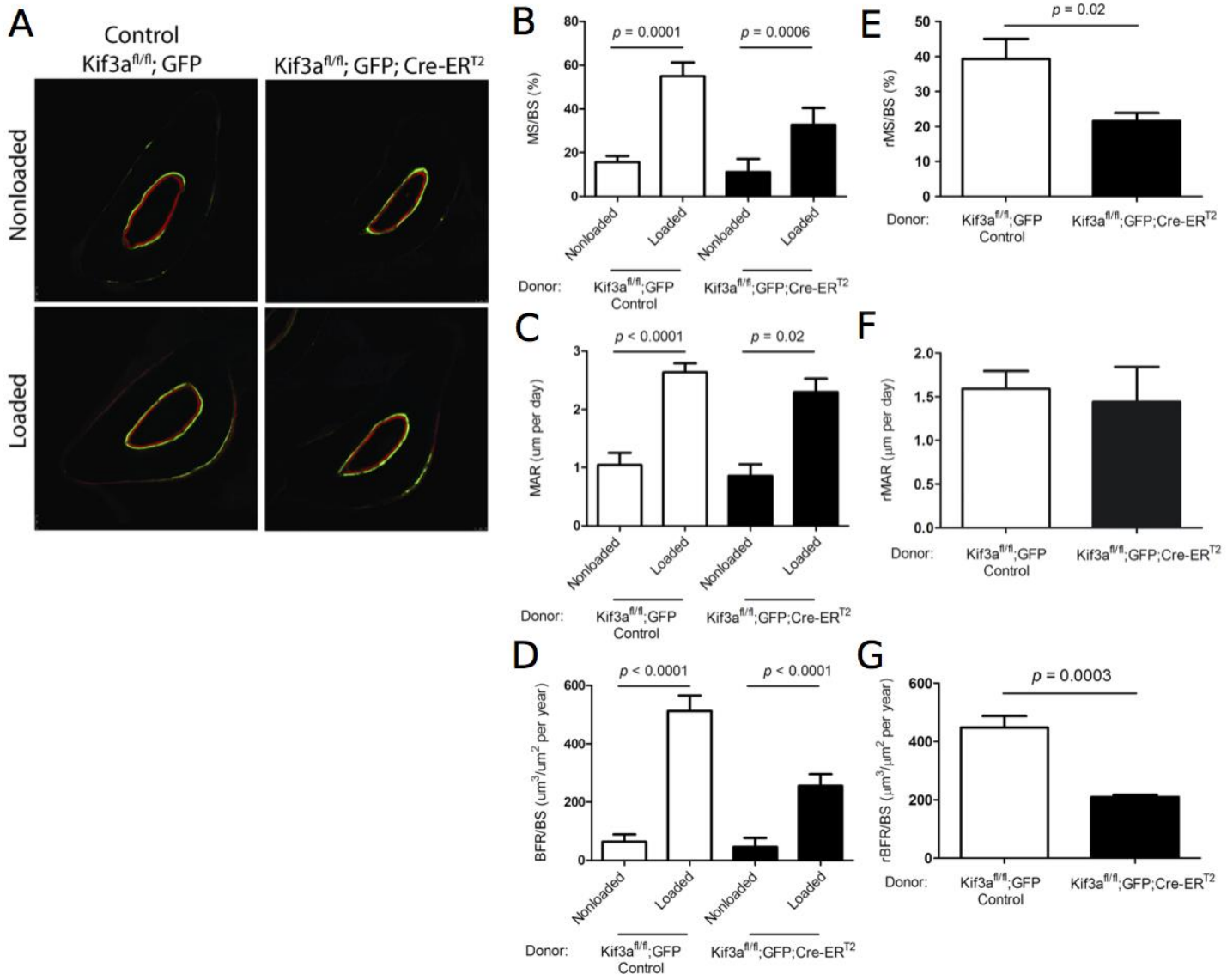

Fig. 3. Primary cilia of progenitor cells mediate mechanically induced bone formation. **(A)** Transverse sections with fluorochrome labels marking new bone formation (green- Calcein, red- Alizarin). Bone formation measures were increased in loaded compared to nonloaded ulnae for control and experimental animals with primary cilia disrupted in bone marrow cells: **(B)** Active mineralizing surface (MS/BS) **(C)** Average interlabel

width, Mineral Apposition Rate (MAR), and **(D)** Total bone formation rate (BFR/BS). Relative measures (r) were obtained by subtracting nonloaded from loaded values: **(E)** rMS/BS, **(F)** rMAR, **(G)** rBFR/BS. Disruption of primary cilia resulted in a diminished response to mechanical loading.