The Role of G Protein-coupled Estrogen Receptor 1 in the Fracture Healing of Normal and Oophorectomized Mice

I. Background/Specific Aims/Hypothesis:

Estrogens are steroid hormones that are known to have functions in the regulation of bone mass and closure of the growth plate.[11] Estrogen also has been shown to play an important role in both early and late stages of fracture healing and as a result, periosteal callus formation is impaired in femur fractures in ovariectomized mice.[1] In these ovariectomized mice, treatment with estrogen or with the selective estrogen receptor modulator, raloxifene, resulted in improved biomechanical properties and a denser trabecular network.[13] However, estrogen and raloxifene had differing effects on healing fractures: raloxifene greatly induced total callus formation, whereas estrogen mainly enhanced new endosteal bone formation. Together, these findings suggested the action of estrogens on fracture healing is complex and requires further studies to determine underlying mechanisms.

G protein-coupled receptor 30 (GPR30), now named G protein-coupled estrogen receptor 1 (GPER1), is a member of the 7-transmembrane receptor family. GPER1 has been shown to bind to 17β-estradiol (E2) with high affinity and potency *in vitro*.[4,9,10,12,14] GPER1 is present in the growth plate chondrocytes, osteocytes, osteoblasts and osteoclasts.[3,6] Animal studies of GPER1 using a mouse with GPER1 deficiency demonstrated some dysregulation of skeletal growth and development which suggests that GPER1 receptor likely plays a role in bone growth, maintenance, and healing.[8,15] Windahl et. al. demonstrated that GPER1 - deficient mice did not exhibit the normal estrogen-induced reduction in femur length and femur growth plate height which suggests that GPER1 plays a role in estrogen-promoted growth plate closure.[15] Recently, Ford *et. al*. studied the effect of GPER1 on osteoprogenitor differentiation.[5] Bone marrow cells from GPER1-deficient mice were cultured *in vitro* under osteogenic differentiation conditions. At 7 days, there was a pronounced decrease in alkaline phosphatase consistent with a decrease in osteoblast number and proliferation.[5] Although these findings demonstrate the significant role of GPER1 in bone remodeling, the effects of GPER1 in fracture healing is not completely clear.

Using an established model of closed femur fractures in mice[7], we propose to test the hypothesis that a *deletion of the GPER1 gene will reduce the ability of the osteoblast to repair a femur fracture.*

We have performed a pilot study with departmental funds in order to ensure the reproducibility of the fracture model in our laboratory (**Figure 1**). In order to reduce the effect of estrogen, mice with oophorectomy will be also used.

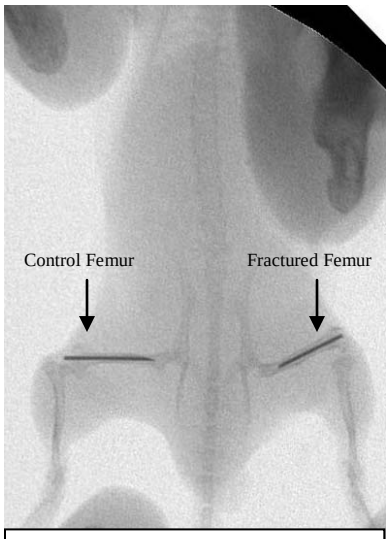


FIGURE 1

The **specific aims** of this project are.

1. To determine the acute (1 week) and long-term (6 weeks) fracture healing responses of the wild-type ($GPER1^{+/+}$) and knockout ($GPER1^{-/-}$ deficiency) mice

2. To determine the long-term effect (6 week) of GPER1 deficiency in bone repair of oophorectomized mice .

The outcomes of this study will be evaluated utilizing radiographs (x-rays), histology, immuno-histochemistry (Type 1, 3 and 10 collagen, Runx 2, RankL) and mRNA levels (Type 1, 2, 3 and 10 collagen, Runx 2, RankL, Rank , OPG, and estrogen, estrogen receptor1&2), and microCT (Bone Mineral Density [BMD]), and biomechanical (torsion) testing.

Our null hypothesis is that the fracture healing response in the GPER $1^{-/-}$ mouse is no different than the wild-type mouse.

II. Significance

With the increase in the elderly population, the management of the elderly patient with the osteoporotic fracture is an ever growing challenge that faces orthopaedic traumatologists. The economic burden of osteoporotic fractures in the United States was estimated to be $17 billion in 2005 and $209 billion over the next decade.[2] Given the enormous impact of osteoporotic fractures, discovering new means to understand, prevent, and better treat these fractures is imperative.

Although past laboratory studies have investigated the effect of estrogen on fracture healing, our study, in contrast, examines fracture-healing mediated by a specific estrogen receptor which is expressed in bone and in the growth plate. This project is relevant and specific to future research and the clinical practice of orthopaedic trauma for two reasons:

1. Characterization of the healing response in GPER1 -deficient mice will provide a better understanding of the influence of estrogen and its role in the pathology associated with osteoporotic fractures.

2. Identification of specific receptors such as GPER1 may provide a future target for pharmacologic modification of fracture healing. For example, it is know that non-specific systemic estrogen-replacement therapy is associated with endometrial cancer in post-menopausal women. By identification and characterization of a receptor such as GPER1, estrogen therapy could be targeted specifically to the bone in order to improve fracture healing and possibly modify osteoporosis, therefore avoiding the ill-effects of non-specific systemic estrogen therapy.

III. Proposed Methods/Plan

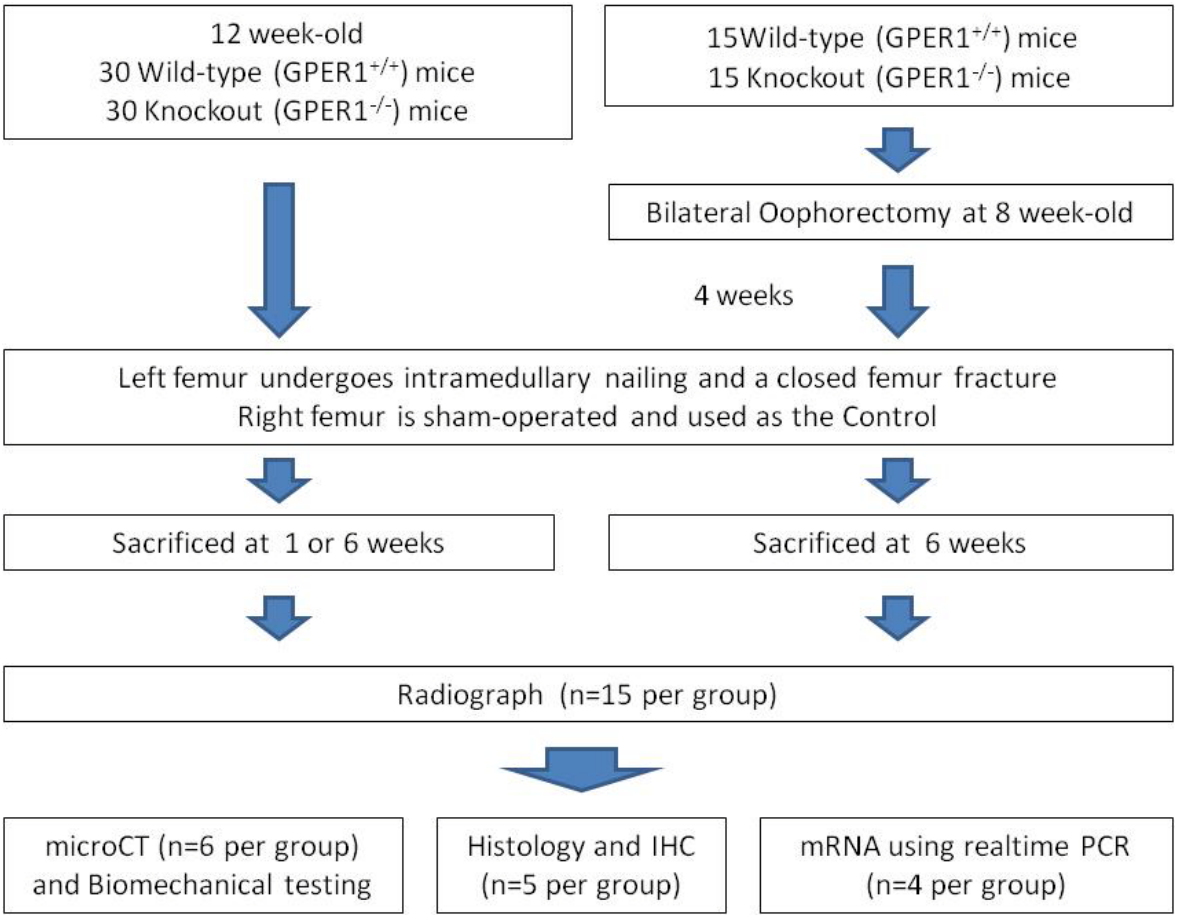
45 wild-type (GPER1 $^{+/+}$) and 45 GPER1 -deficient (GPER1$^{-/-}$) 12-week-old female mice will be provided by Dr. Orhan Oz (see Reference 13) in the study. Fifteen of the wild-type and 15 GPER1 -deficient (GPER1$^{-/-}$) mice will undergo total bilateral oophorectomy (Day -28) at eight weeks of age, prior to creation of the fracture.

At experimental Day 0, the left femur of each animal will undergo intramedullary nailing and creation of a closed femur fracture and the right femur will be sham-operated and used as the control. In each experimental group, 15 animals will be randomly selected for oophorectomy. The ovariectomized group will be evaluated only at Day 42.

At each time point, the selected cohort of 15 animals will be sacrificed and placed into three groups. Group 1 will be utilized for microCT and biomechanical testing. Group 2 will be utilized for fracture callus assessment (histologically evaluated for the presence of inflammatory response, mesenchymal cell infiltration, chondrocyte appearance, callus/bone formation and microCT). Finally, in the third group we will utilize qPCR to measure the fracture healing response (quantitative measurement of markers such as expression of Types III and X collagen, expression of Rank ligand, expression of Runx2).

The study protocol is summarized below in **Figure 2**.

FIGURE 2. Study Protocol



Outcomes:

1. Radiographic analysis (plain radiographs (n=15/group), microCT (n=6/group) of callus will allow for quantitative analysis and comparison of fracture healing between wild-type, oophorectomized, and GPER1-deficient mice.

2. Histological (n=5) and biomechanical evaluation (n=6 evaluated after microCT) at each time point will be analyzed and will allow for further comparison between the GPER1 knockout, oophorectomized, and wild-type mice fracture healing.

3. The expression of type 1, 2, 3 and 10 collagen, Runx 2, RankL, Rank , OPG, estrogen, estrogen receptor1 and estrogen receptor 2), at each time-point (n=4)will be normalized to the GAPDH or β-actin and will be statistically analyzed. In situ (localized) changes of collagen 1&3, RankL, Rank and OPG along with estrogen will also be analyzed using immunohistochemistry.

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