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A potential role for Dkk-1 in the pathogenesis of osteosarcoma predicts novel diagnostic and treatment strategies.

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Canonical Wnt signaling is an osteo-inductive signal that promotes bone repair through acceleration of osteogenic differentiation by progenitors [1-12]. Dkk-1 is a secreted inhibitor of canonical Wnt signaling [13-17] and thus inhibits osteogenesis [18-21]. To examine a potential osteo-inhibitory role of Dkk-1 in osteosarcoma (OS), we measured serum Dkk-1 in pediatric patients with OS (median age, 13.4 years) and found it to be significantly elevated. We also found that Dkk-1 was maximally expressed by the OS cells at the tumor periphery and *in vitro* Dkk-1 and RANKL are co-expressed by rapidly proliferating OS cells. Both Dkk-1 and conditioned media from OS cells reduces osteogenesis by human mesenchymal cells and by immuno-depletion of Dkk-1, or by adding a GSK3β inhibitor, the effects of Dkk-1 were attenuated. In mice, we found that the expression of Dkk-1 from implanted tumors was similar to the human tumor biopsies in that human Dkk-1 was present in the serum of recipient animals. These data demonstrate that systemic levels of Dkk-1 are elevated in osteosarcoma. Furthermore, the expression of Dkk-1 by the OS cells at the periphery of the tumor probably contributes to its expansion by inhibiting repair of the surrounding bone. These data demonstrate that Dkk-1 may serve as a prognostic or diagnostic marker for evaluation of OS and furthermore, immuno-depletion of Dkk-1 or administration of GSK3β inhibitors could represent an adjunct therapy for this disease.

We measured the levels of Dkk-1 in the serum of newly diagnosed individuals with OS by ELISA and found that, the mean levels were elevated ($p<0.0001$, 2 sample t-test) in affected individuals (range, 16.84 ng mL^{-1} to 2210.14 ng mL^{-1}, mean 191.91 ng

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mL^{-1}, median 90.53 ng mL^{-1}) when compared to unaffected individuals (range, 2.28 ng mL^{-1} to 43.38 ng mL^{-1}, mean 21.66 ng mL^{-1}, median 19.67 ng mL^{-1}). Although the control group (n=12) was smaller than the OS group (n=37), and the median age of the unaffected individuals was slightly higher (by approximately 7 years), the control Dkk-1 values were similar to the normal levels reported by Tian *et al.* [20] who demonstrated that elevated levels of serum Dkk-1 were co-incident with the osteolytic lesions seen in most cases of multiple myeloma (**Fig.1a**). The Dkk-1 levels in the affected individuals were somewhat higher than those documented in the Tian *et al.* study with the highest levels in the micromolar range. Immunohistochemical staining of excised tumor biopsies demonstrated that Dkk-1 was expressed maximally at the periphery of the tumor, adjacent to the hosts' bone tissue (**Fig.1b**). The expression of Dkk-1 by two osteosarcoma cell lines; MG63, a well-characterized osteogenic sarcoma and LS1, a cell line derived from an excised osteosarcoma, was examined in more detail in tissue culture experiments. Dkk-1 was found to be maximally secreted by cells rapidly proliferating in sparsely populated monolayers but was significantly reduced as proliferation slowed and the monolayer became more confluent (**Fig.1c-d**). Interestingly, Dkk-1 expression in the higher density monolayers was confined to a small fraction of cells that were clearly in the metaphase of division (**Fig.1d**). Also the potent upregulator of osteoclast activity, RANK ligand (RANKL) [22] mirrored the expression of Dkk-1 in OS cells but it was exclusively detected as the membrane bound form rather than the secreted form (**Fig.1e,f**). Based on these observations, we hypothesized that the expression of Dkk-1 and RANKL at the periphery of the tumor was necessary for osteogenic remodeling as the tumor expands. The presence of high levels of Dkk-1 and RANKL facilitate

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expansion by allowing the proliferative cells at the periphery of the tumor to accelerate bone resorption through expression of RANKL whilst inhibiting osteoid repair through the action of Dkk-1.

The putative osteo-inhibitory effect of recombinant Dkk-1 was tested in a tissue culture model of osteogenic differentiation by human primary mesenchymal stem cells (MSCs) [21]. At concentrations equivalent to those measured in OS patients, Dkk-1 inhibited the expression of the osteogenic marker, alkaline phosphatase, by MSCs in a dose dependent manner when added to osteogenic cultures (**Fig.2a**). The effect was observed in MSCs from 3 donors and pooled murine MSCs. In 2 donors (**Fig.2a**), MSCs cultured directly from bone spicules (**red**) were more resistant to Dkk-1 than those cultured from the fluid component of the marrow (**black**) suggesting that the MSCs were probably osteogenically pre-conditioned by the niche of the bone tissue. The observation that serum alkaline phosphatase is occasionally upregulated in osteosarcoma patients, suggests that Dkk-1 acts to prevent differentiation of progenitor cells, but does not affect the release of alkaline phosphatase from pre-existing osteoblasts at sites of bone remodeling.

When media was conditioned by MG-63 OS cells and added to osteogenic cultures of MSCs, osteogenic inhibition occurred (**Fig.2c**) and this effect was attenuated upon immuno-depletion of Dkk-1 from the medium (**Fig.2b,c**). Dkk-1 inhibits the Wnt pathway by sequestering the Wnt co-receptor, LRP6 and preventing the Wnt-induced coalescence of Frizzled and LRP6 at the membrane. The downstream effect of the LRP6/Wnt/Frizzled complex is to inhibit glycogen synthetase kinase 3 beta (GSK3β), reduce phosphorylation of β-catenin and prevent its degradation by the

proteosomal machinery. Stabilized β-catenin complexes with TCF/LEF mediates transcription of target genes, in this case, osteogenic genes. The presence of a pharmaceutical inhibitor of GSK3β would be predicted to elicit the same effect as Wnt signaling, irrespective of the level of Dkk-1 in the system. Osteogenic cultures were prepared in the presence of Dkk-1 with or without the GSK3β inhibitor, bromo-indirubin-3'-mono-oxime (BIO). The presence of BIO reduced the osteo-inhibitory effect of Dkk-1 (**Fig.2d**). Since Wnt signaling has been implicated in the induction of oncogenesis, we tested the effect of escalating doses of BIO on MG-63 and LS-1 cell proliferation. At the concentrations tested, there was no significant induction of proliferation by BIO (**Fig.2e**).

We established an osteosarcoma model to recapitulate some of the effects of Dkk-1 and OS *in vivo*. MG-63 cells were labeled by lentiviral transduction of a fusion gene combining the mitochondrial localization sequence of cytochrome c oxidase with the fluorescent protein, dsRed (**Fig.3a,b**). Upon suspension culture in the presence of clotted human plasma, after 24-48 hours the cells formed tumor spheroids that ranged from approximately 10 to 5000 cells in diameter within the fibrin gel. Smaller spheroids expressed Dkk-1 throughout (**Fig.3d**), but the larger structures adopted an expression pattern for Dkk-1 that mimicked the tumor biopsies (**Fig.3e**) with the maximal level of expression at the periphery. To examine the expression and distribution of Dkk-1 expression by osteosarcoma cells *in vivo*, fibrin constructs containing 1 million and 10 million labeled MG63 cells were implanted in nude mice against the upper thoracic vertebrae. After 1 week, the constructs were clearly visible by live animal fluorescent imaging (**Fig.4a**). Furthermore, human Dkk-1 could be detected in the blood of implanted animals when assayed by ELISA (**Fig.4b**) and the level of circulating Dkk-1 correlated

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with the number of surviving cells in the construct. After 2 and 4 weeks post implantation, the number of MG63 cells present in the recipients had reduced resulting in a concomitant reduction of systemic Dkk-1 (data not shown). The reason for the reduced viability of the cells over extended implantation periods is unclear but macrophage mediated destruction of implants in immuno-compromised mice has been reported in the literature [23]. In spite of the hosts' response to the implanted cells, human Dkk-1 could be detected in the blood of the recipient mice, demonstrating that tumor-derived Dkk-1 escapes into the blood stream. It is unclear at this point whether the elevated systemic Dkk-1 in OS patients is derived solely from the tumor, since the human Dkk-1 circulating in the blood of recipient mice was much lower than the mean levels detected in the blood of the human OS patients. It is possible, however, that the host tissue interacts with the tumor resulting in upregulation of the expression of Dkk-1, a phenomenon observed in the case of multiple myeloma [21]. The host microenvironment in the patients may be more readily affected by the tumor than the surrounding mouse tissue accounting for the reduced levels of Dkk-1 in the mouse blood when compared to the human blood. At any rate, the presence of OS cells in both humans and the recipient mice resulted in elevated circulating human Dkk-1, suggesting that the molecule could represent a valuable diagnostic tool. The correlation of tumor load with Dkk-1 levels also suggests that the assay also has potential for measuring the relative size and severity of such tumors.

These data strongly suggest that the secretion of the canonical Wnt inhibitor Dkk-1 is highly expressed by OS tumors at levels that become detectable in the systemic circulation in humans. Furthermore, the *in vivo* data demonstrates that the level of Dkk-1 detectable in blood is proportional to the number of surviving OS cells in the tumor.

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Assays of Dkk-1 secretion could therefore represent a useful diagnostic and prognostic tool for the evaluation of OS patients. Serum Dkk-1 measurements may be useful for the evaluation of other types of malignancy but presently it is unclear whether the expression is confined solely to osteolytic tumors. This certainly seems feasible since a recent study has demonstrated that prostate tumors that express high levels of Dkk-1 produce more extensive local bone destruction [24]. Reducing the osteoinhibitory effects of Dkk-1 would be predicted to reduce local bone damage, and as a result, probably reduce the expansion of the tumor. This could be achieved by pharmaceutical inhibition of GSK3β or by antibody mediated sequestration of Dkk-1. The osteoinductive properties of GSK3β inhibitors have been demonstrated both *in vivo* and *in vitro* by numerous investigators [25] and the benefits of administration of an anti-Dkk-1 antibody has been demonstrated recently in a murine model of multiple myeloma [26]. However, it remains to be seen whether the induction of Wnt signaling either by GSK3β inhibition, or by antibody administration may affect the metastatic potential of OS cells since Wnt signaling and/or beta catenin upregulation has been shown to be a key regulator of migration in prostate tumors, multiple myeloma cells and also in OS cells [24, 27, 28]. Since tumor derived Dkk-1 was present in the blood of mice at levels proportional to the number of surviving tumor cells, Dkk-1 may also serve as a prognostic or diagnostic marker for evaluation of patients with OS.

Materials and methods.

Human biomaterial acquisition. The handling and acquisition of human derived biomaterials were performed in accordance with the institutional review boards of Tulane

University Hospital and Clinic (New Orleans, Louisiana, USA) and St. Jude Children's Hospital (Memphis, Tennessee, USA). The OS serum samples were acquired from the Tissue Bank of St. Jude Children's Hospital, and the control group samples were collected from unaffected individuals at Tulane University Hospital and Clinic. Human mesenchymal stem cells were acquired from the Tulane Adult MSC Distribution Core (Tulane University, New Orleans, LA) and cultured in accordance with their protocols.

ELISA assays. Frozen serum samples from newly diagnosed patients with OS were acquired from St. Jude Children's Hospital under the supervision of Dr. N. Daw and Dr. E. Horwitz. Serum samples from unaffected individuals were drawn and prepared at Tulane University Hospital and Clinic. ELISA assays were performed as previously described [21].

Histology and immunocytochemistry. Osteosarcoma tumors, synthetic tumor constructs or monolayer cells were fixed in formalin and processed as paraffin blocks if necessary. For histochemistry, 8 μm sections were prepared, deparaffinized and rehydrated. After an acidic antigen retrieval step (R and D Systems, Minneapolis, MN), sections were blocked and incubated in the presence of a 1:800 dilution of goat anti-human Dkk-1 or monoclonal anti RANKL antibody (R and D Systems). Monolayer cultures were directly subjected to immunocytochemistry after fixation. Alexafluor 594 or 488 conjugated secondary (Invitrogen, Carlsbad, CA) antibodies were employed to detect antigen binding.

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Alkaline phosphatase assays. Monolayer phosphatase assays were performed on monolayers in 6-well format as previously described [21]. Dkk-1 preparation and culture conditions were as previously described [21]. BIO was acquired commercially (Calbiochem, LaJolla, CA).

Cell counting assays. Cells were counted based on nucleic intercalation of a fluorescent dye (CyQuant, Invitrogen). Assays were performed as previously described [21, 29].

Western blotting. Western blots were performed on Triton X100 (Sigma, Poole, UK) insoluble extracts of cells using the goat anti-human Dkk-1 or monoclonal anti RANKL antibody (R and D Systems). Controls for actin and GAPDH were employed as previously described [30].

Immunoaffinity depletion. Dkk-1 was depleted from the medium by antibody incubation and protein A mediated depletion as previously described [29].

Cell labeling. The lentiviral construct encoding the dsRed fluorescent protein coupled to the mitochondrial localization sequence of human cytochrome C oxidase subunit VIII was prepared using standard protocols by virus core facility at Louisiana State University viral vector core [31,32]. Proliferating MG63 cells were exposed to the virus at a multiplicity of infection of 80 in the presence of 9 μg mL^{-1} polybrene for 18 hours. After 4 days, approximately 50% of the cells expressed the fluorescent protein. Expressing

cells were selected by fluorescent activated cell sorting (Facsvantage SE, Becton Dickinson).

Constructs. Labeled cells were suspended in 1 mL of a 2x reconstitution of dried human plasma (Sigma) and mixed with an equal volume of thromboplastin C (Plastinex, Fisher Lifesciences, Pittsburg, PA). The mixture was transferred to a 10 mm x 20 mm chamber slide for gelling. Clotting was allowed to proceed for 2-4 hr, and then the appropriate experimental medium preparation was added to cover the solid construct until implantation.

Implantation. Fibrin constructs were implanted subcutaneously between the scapulae of anaesthetized nude mice. A 10 mm incision was made longitudinally between the scapulae and a small cavity was made between the dermis of the skin and the fascia below to accommodate the constructs which were 10 mm square. The incision was then closed by 2-3 sutures, and sealed (Vetbond, 3M, St. Paul, MN). After 5 days, the sutures were removed. Seven days thereafter, the animals were placed under anesthesia, euthanized by cardiac exsanguination, and serum was prepared from the blood. The implants were removed for genomic DNA extraction. Genomic DNA was extracted from the tissue by phenol chloroform extraction (Trizol, Invitrogen) and subjected to quantitative real time PCR for the dsRed gene using the following primers: forward; ACTACAAGAAGCTGTCCTTCC and reverse; TTCACGCCGATGAACTTCACC. Reactions were cycled on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) for 40 cycles with the annealing temperature set to 60^{o}C. Products were detected by

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fluorescence intercalation (SYBR Green, Applied Biosystems) and validated by gel electrophoresis and melting curve analysis.

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Figure legends.

Figure 1: Panel a: Scatter plot of the circulating Dkk-1 levels in OS patients and unaffected individuals. Measurements were performed by ELISA assay. For statistical values, see text. **Panel b:** A sectioned osteosarcoma immuno-stained for the detection of Dkk-1 (red). Dkk-1 expression is maximal at the border zone between the osteoid tissue (green autofluorescence) and the tumor cells (white dotted line). Nuclei are stained with DAPI (blue) **Panel c:** Secretion of Dkk-1 by rapidly dividing, low density cultures (log) and slowly dividing confluent cultures (slow) of MG63 and LS1 osteosarcoma cells. Measurements were achieved by ELISA assay, values represent the mean (n=6), and error bars represent standard deviations. P values were calculated by 2-tailed Student's t-test. **Panel d:** Monolayers of MG63 cells at high (slow) and low (log) density immunostained for Dkk-1. Note that staining is maximal in the low density cultures and in the high density cultures, Dkk-1 staining is confined to those cells undergoing mitosis (arrowed). The DNA is stained with DAPI (blue). **Panel e:** Western blot of membrane isolates derived from MG63 and LS1 osteosarcoma cells. The control lanes (upper) were simultaneously incubated with an anti-β-actin and anti-GAPDH antibodies since enrichment for insoluble, membrane bound, β-actin confirms that the membranes have been efficiently recovered at the expense of cytosolic components such as GAPDH. Membrane bound RANKL was detected on the same stripped blot (lower) with an anti RANKL antibody. **Panel f:** Monolayers of non-permeablised MG63 cells at high (slow) and low (log) density immunostained for RANKL. Note that staining is maximal in the low density cultures and punctuate in the high density cultures. The DNA is stained with DAPI (blue).

Figure 2: Panel a: Osteogenic differentiation of MSCs in the presence of Dkk-1. Results from cells derived from 3 human donors and pooled murine donors are presented. Osteogenic differentiation is presented as a function of membrane ALP activity, an early marker of osteogenesis. Measurements are normalized to control levels of activity, designated 1.0. The black lines represent MSCs prepared from the fluid component of bone marrow and the red lines represent MSCs prepared from bone spicules filtered from the aspirates. Dkk-1 exposure causes a dose dependent inhibition of alkaline phosphatase activity. **Panel b:** Immunodepletion of Dkk-1 from MG63 OS conditioned medium through incubation with a polyclonal antibody against Dkk-1. The Dkk-1:antibody complexes were removed from the medium by protein A affinity chromatography, then the medium was assayed by ELISA. **Panel c:** Osteogenic differentiation by MSCs in the presence of non-depleted and Dkk-1 immuno-depleted conditioned medium from MG63 OS cells. Representative results from one of three donors are presented. Measurements were achieved by ALP assay, values represent the mean (n=6), and error bars represent standard deviations. P values were calculated by 2-tailed Student's t-test. **Panel d:** Osteogenic differentiation by MSCs in the presence of Dkk-1 and with or without the GSK3β inhibitor BIO. **Panel e:** The effect of a range of BIO doses on the proliferation of osteosarcoma cells. Cell numbers were evaluated by fluorescent nucleic acid intercalation assay.

Figure 3: Panel a: Fluorescence activated cell sorting of transduced cells expressing mitochondrially localized red fluorescent protein. The cells from the gate designated M1 were used in subsequent experiments. **Panel b:** Micrographs of the labeled MG63 OS cells. **Panel c:** Micrographs of the tumorspheres derived from culture in clotted human plasma. **Panel d:** A sectioned tumorsphere (red) immuno-stained for the detection of Dkk-1 (green). Nuclei are stained with DAPI (blue). The isotype control is presented on the right.

Figure 4: Panel a: Live animal fluorescence imaging of an implanted construct containing labeled MG63 OS cells. **Panel b:** Evaluation of human Dkk-1 levels in the blood of implanted animals after 1 week. The x-axis represents the initial number of implanted cells. Measurements were achieved by ELISA assay on mouse serum, values represent the mean (n=4, 2 males and 2 females), and error bars represent standard deviations. P values were calculated by 2-tailed Student's t-test.

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Figure 1

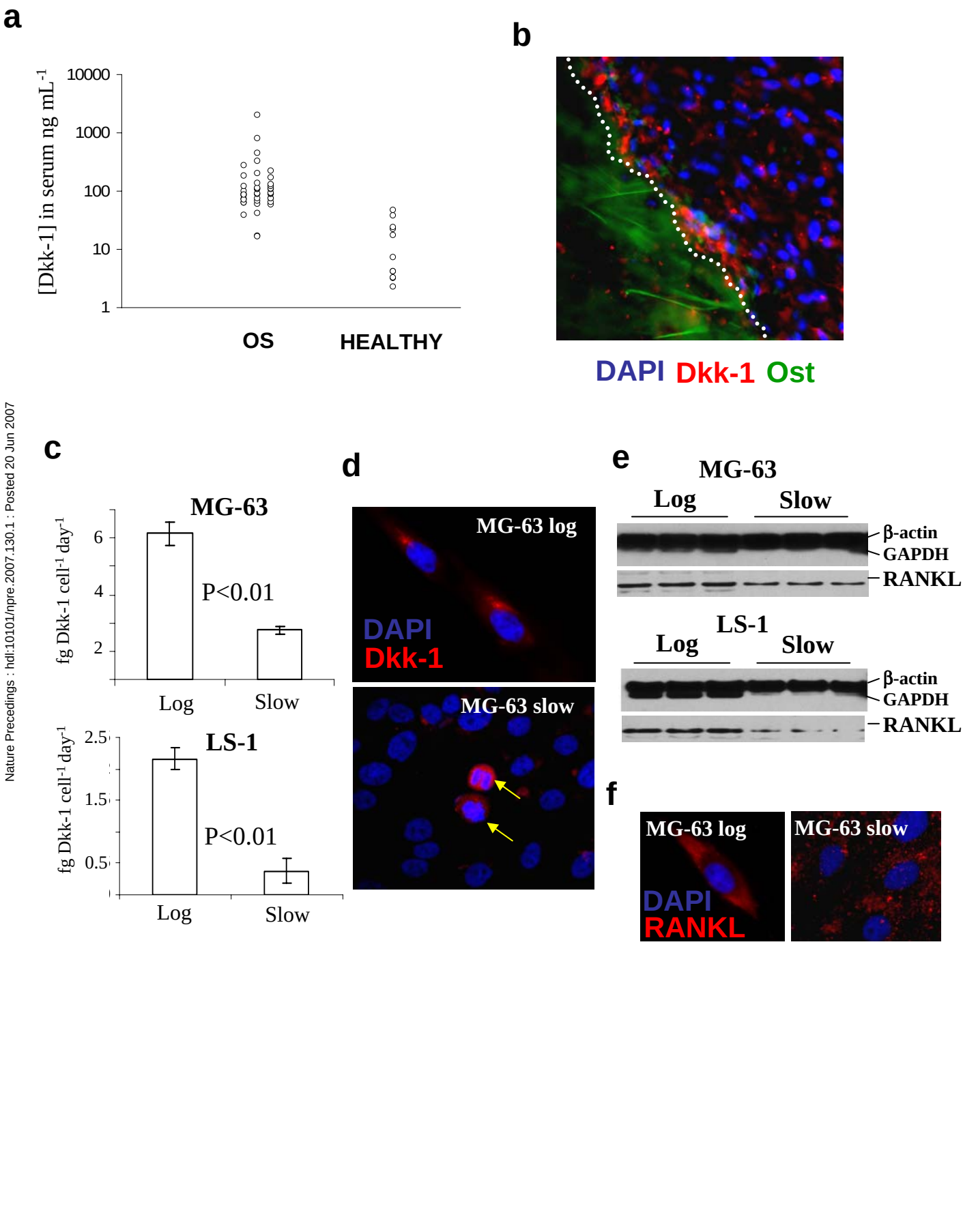

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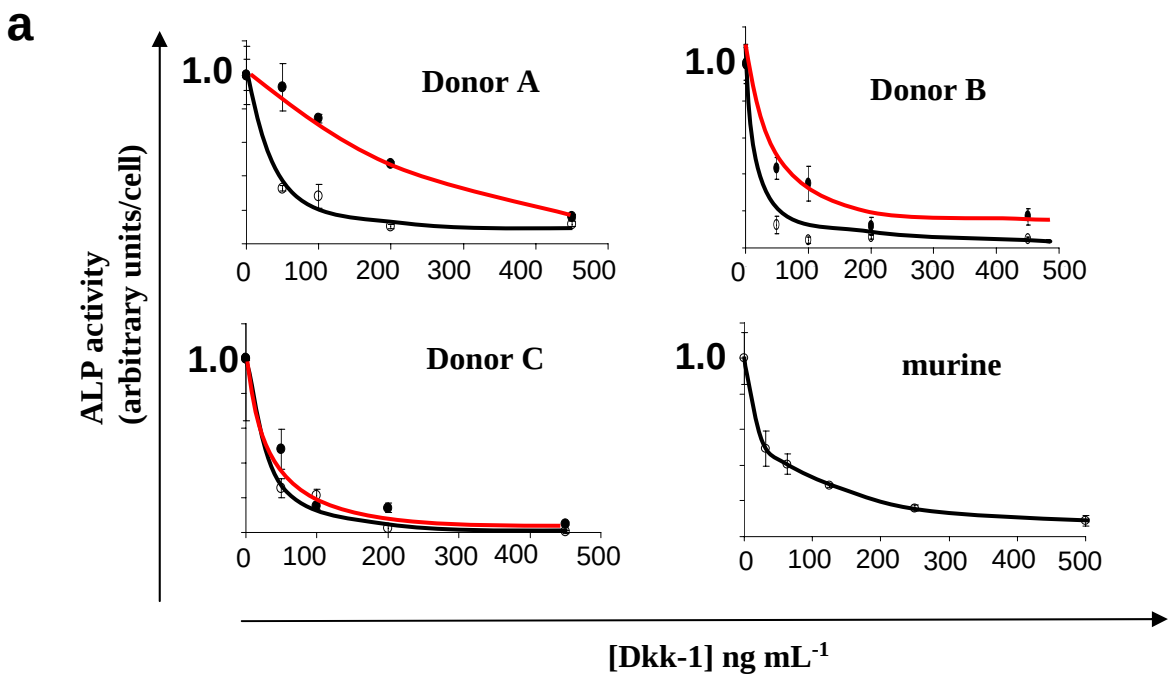
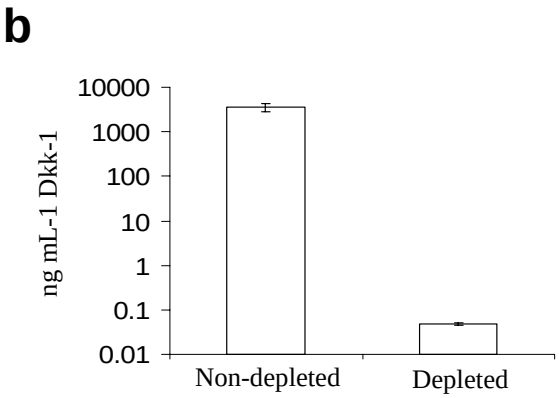
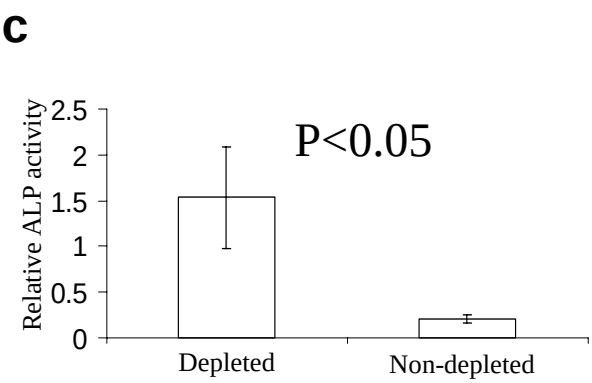
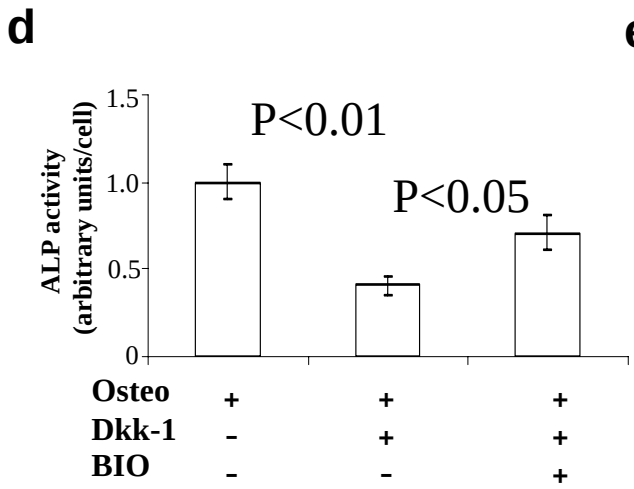
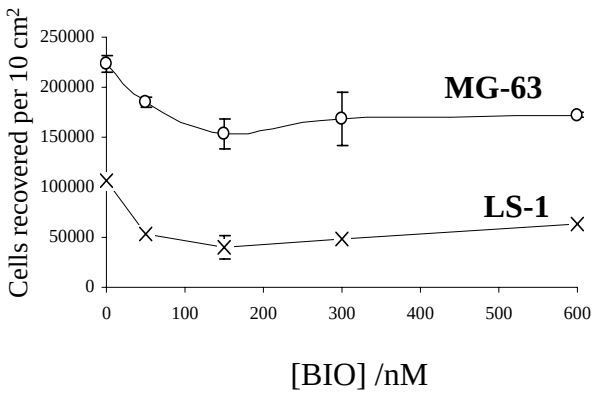
Figure 2.





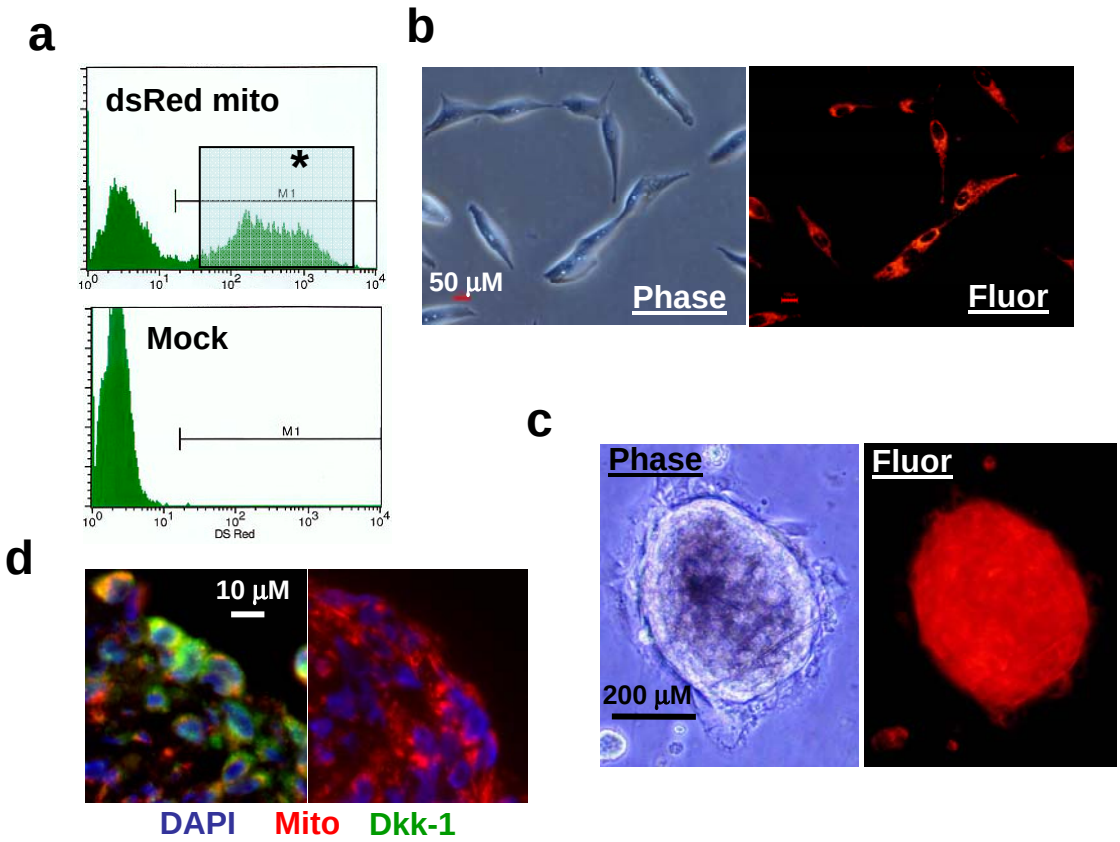






Nature Precedings : hdl:10101/npre.2007.130.1 : Posted 20 Jun 2007

Figure 3.



Nature Precedings : hdl:10101/npre.2007.130.1 : Posted 20 Jun 2007

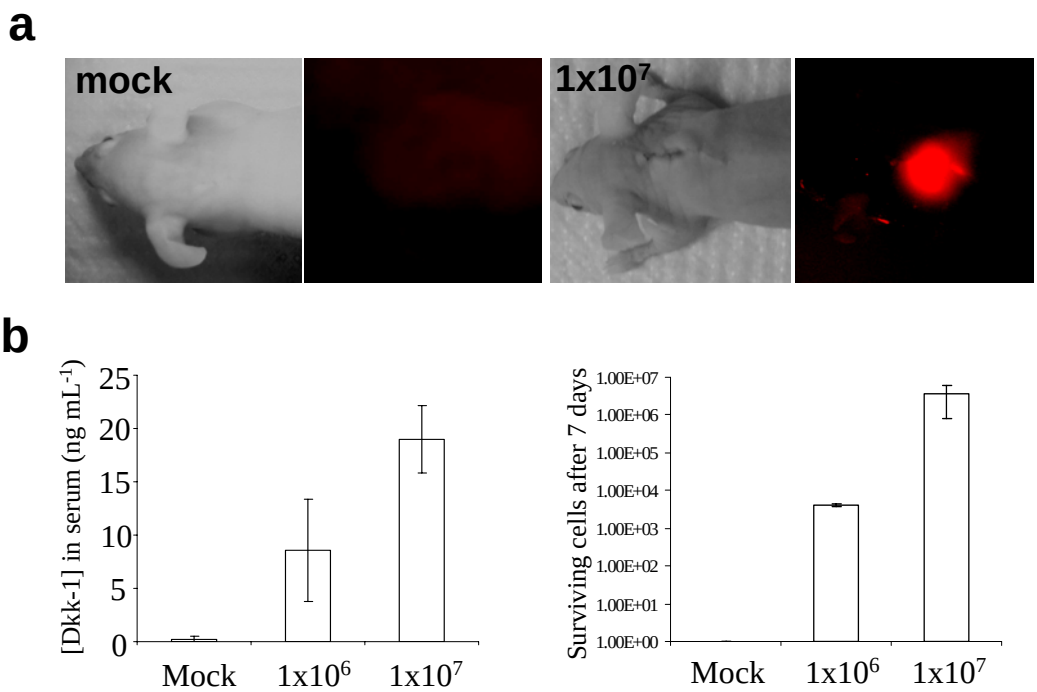



Figure 4.