Dexamethasone Exposure Accelerates Endochondral Ossification of Chick Embryos Via Angiogenesis

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ABSTRACT

Dexamethasone (Dex) is widely used to treat chronic inflammatory diseases in the clinic. Increasingly, there is more attention being paid to the side effect of Dex. In this study, we investigated the involvement and mechanism of Dex exposure in accelerating mineralization during long bone formation. We first determined that Dex exposure could accelerate long bone mineralization in vivo, but there was no apparent difference between control and Dex-treated in the phalanges model in vitro. Next, we established that Dex exposure promoted angiogenesis in the chick yolk sac membrane model. In addition, it increased human umbilical vein endothelial cell proliferation and migration in culture. We found that Dex could enhance angiogenesis when phalanges were cultured on chick chorioallantoic membrane and correspondingly increased the expression of angiogenesis-related genes in the phalanges. Furthermore, we also revealed that Dex exposure reduced the number of osteoblasts and simultaneously increased the number of osteocytes in ex vivo-cultured phalanges. Runx-2 and Col10α1 expressions were up-regulated by Dex exposure, indicating that Dex exposure accelerated the terminal differentiation of osteoblasts. Lastly, we demonstrated that MC3T3-E1 cells cultured in the presence of Dex accelerated their mineralization. In summary, we have shown that the ability of Dex to initiate angiogenesis is the mechanism that allows it to accelerate mineralization during long bone formation.

Key words: dexamethasone; chick embryos; mineralization; angiogenesis; osteogenesis

Dexamethasone (Dex) is a potent synthetic member of the glucocorticoid (GC)-like family of steriod hormones. It has been widely used in the clinic to relieve inflammations including certain kinds of arthritis, severe allergies, asthma, and treatment of certain cancers. GCs synthesis is regulated by the hypothalamic-pituitary-adrenal axis in response to organic metabolism requirement. Endogenous GCs are indispensable for bone formation, osteoblast differentiation, and maintenance of bone structure during embryonic osteogenesis (Mushtaq et al., 2002). However, when GCs concentration is higher than physiological levels, it affects osteoblast differentiation and lead to GCs-induced osteoporosis (Ishida and Heersche, 1998). In addition to hormones, paracrine factors such as insulin-like growth factor and NF-κB expression also play important roles (Carbone et al., 2012; Joanny et al., 2012). These hormones and bioactive molecules are closely associated with bone homeostasis, ie, balancing bone resorption by osteoclasts and new bone formation by osteoblasts. Any imbalance in these processes could lead to
bone being lost, especially by factors that inhibit new bone forma-
tion and stimulate bone resorption (Olney, 2009). It is gener-
ally known that GCs are teratogenic and causes cleft palate to
develop if used indiscriminately—as revealed in animal models
(Azziz and Ladda, 1990). Therefore, Dex application in clinic has
attracted public concern about its teratogenicity in human em-
byo development (Meyer-Bahlburg et al., 2004). However, it re-
mains obscure on how Dex exposure during pregnancy affects
osteogenesis in the developing embryo.

In vertebrates, long bone development starts by mesenchy-
mal cells in the limb bud condensing and undergoing
chondrogenesis to form a hyaline cartilage model with the
shape of the perspective bone. These cartilaginous anlagen are
then calcified to form compact bone and the bone marrow.
The limb long bones, vertebrae, ribs, and pelvis are formed by
endochondral ossification—based on a pre-existing hyaline
cartilage model. In our previous study, we have demonstrated
that Dex exposure exerted a biphasic effect on the skeletal
structure of embryonic long bones. In this context, we want to
further investigate the mechanism of Dex-induced abnormal
osteogenesis.

It is well known that bone tissue has a well-established vas-
cular system and angiogenesis plays a crucial role in skeletal
development and repair (Kanczler and Oreffo, 2008). One of the
features of endochondral ossification process is the invasion of
the cartilage by blood vessels which replaces the cartilage tem-
plate with bone tissues (Kronenberg, 2005; Provot and Schipani,
2005). Angiogenesis involved the production of new blood ves-
sels from a pre-existing vasculature. It is under the modulation
of pro- and anti-angiogenic signaling molecules during embryo
development (Carmeliet, 2003; Polverini, 2002). During the adult
period, angiogenesis is limited to a few physiological processes
such as endometrial angiogenesis following menstruation and
pathophysiological process such as cancer, wound healing, ar-
thritis, and psoriasis.

Vascular endothelial growth factor (VEGF)-A is one of indis-
ispensable regulators of blood vessel invasion in the cartilage
model. VEGF-A is synthesized and released by chondrocytes
during chondrogenesis and osteoblasts at later stages of differ-
etiation (Pfander et al., 2004; Zeiler and Olsen, 2005). Its recep-
tors are VEGF-R1 (Flt-1) and VEGF-R2 (KDR/Flik-1) involved in
regulating both physiological and pathological angiogenesis
during embryonic osteogenesis (Shibuya, 2006). In addition,
many other signaling molecules including hypoxia-inducible
factor 1α and parathyroid hormone-related protein play vital
roles during the mutually dependent osteogenesis and angi-
genesis (Bentovim et al., 2012; Kigami et al., 2013).

The chick chorioallantoic membrane (CAM) is a widely used
model for studying angiogenesis (Siambis et al., 1996). This is
because CAM is a highly vascularized membrane and its posi-
tion directly under the inner surface of egg shell—making it eas-
ily accessible to experimentation. CAM has been used to sup-
sort xenograft development because CAM could supply
blood vessels to the xenograph. Another model for studying
angiogenesis is the yolk sac membrane (YSM). The chick embry-
onic yolk sac is an extra-embryonic structure and the first site
where blood vessels and hematopoietic cells develop. During
embryo development, the chorionic membrane and allantois
become fused together to form the CAM. The YSM is also an ex-
cellent model for studying blood vessel formation (Costa et al.,
2013), because it is highly vascularized, results are easily repro-
ducible, it is simplistic, and low-cost to use. In this study, we
have used these models and cell cultures to investigate the ef-
ects of Dex on angiogenesis and osteogenesis.

MATERIALS AND METHODS

Embryo manipulation. Fertilized Leghorn eggs were obtained from
the Avian Farm of the South China Agriculture University
(Guangzhou, China) and incubated in a humidified incubator
(Yiheng Instruments, Shanghai, China) at 38°C until the
embryos reached the desired developmental stage (Hamburger
and Hamilton, 1992). The chick embryos were exposed to differ-
cent concentrations of Dex (10⁻⁷, 10⁻⁸, or 10⁻⁹M) or control 0.1%
DMSO (Sigma-Aldrich, Missouri, USA) for 7.5 days as previously
described (Cheng et al., 2012). In brief, ~100 µl of DMSO or 10⁻⁷–
10⁻⁹M Dex were carefully injected into a small hole made in the
air chamber of the egg on 1.5 day of incubation. After the treat-
ment, the embryos were further incubated for 7.5 days before
being harvested for analysis.

Alcian blue and alizarin red staining. To visualize the skeleton, the
chick embryos were stained with alcian blue and alizarin red
dyes as previously described (Schmitz et al., 2010). Nine-day-old
chick embryos were fixed in 95% ethanol for 3 day, the skin and
viscera were then carefully removed and post-fixed for one
more week. The embryos were stained in 0.1% alcian blue and
alizarin red (Solarbio, Beijing, China) dyes in 70% ethanol for 1
week and then cleared in 25% glycerol/1% KOH for 3 days.
Finally, the embryos were treated in a graded series (75%–100%)
of glycerol. To make parts of the skeleton more visible, we
carefully dismembered long bone tissues and photographed
them using a stereomicroscope (Olympus MVX10, Japan). The
length of the alizarin red stained portion of each humerus,
radius, and femur were quantified and analyzed using the
Image Pro-Plus 5.0 (IPP, Media Cybernetics, Rockville, Maryland)
software.

Phalanx explant cultures. The fertilized eggs were incubated for 17
days before similar segments of phalanges were isolated and
randomly used as control or Dex treatments. The phalanges
were cultured in DMEM (GIBCO, New York) supplemented with
10% FBS (GIBCO) containing Dex or 0.1% DMSO (control) at 37°C
and 5% CO₂ (Galaxy S, RS Biotech, UK). The cultured phalanges
were examined using an inverted microscope (Nikon Eclipse Ti-
U, Japan) and the lengths and degree of mineralization of the
phalanges were measured 1–8 days after the treatment with
Dex. The growth rate of phalanges was quantified according to
the ratio of the increasing length of first day to 0 day to the
absolute value of the full length of phalanges on 0 day, and so
forth for the 8 days, respectively. Mineralization of the cultured
phalanges was quantified by the ratio of the dark area of each
phalanx to the overall whole bone area. Each treatment was
performed in triplicates.

Angiogenesis assessment on YSM. Angiogenesis in YSM was
assessed as previously described. In brief, fertilized eggs
were incubated for 2.5 days and then placed into a sterilized
glass dish with the YSM facing upward. Two silicone rings
were placed on top of the leading edge of the blood vessels
marked with ink to indicate the starting position of the YSM
within the ring. 50 µl of 0.1% DMSO (control) was introduced
into the ring located on the left side of the YSM, marked in
red. While 50 µl of 10⁻⁶ M Dex was introduced into the ring
marked in black on the right side of the same embryo. The
time of expansion of the blood vessel plexus inside the sil-
cone rings was determined and photographed after 12–36 h
incubation. Ten embryos in each treatment group were
examined.
The blood vessels density (BVD) in the YSM was analyzed using the Image Pro-Plus 5.0 software. The BVD was expressed as the percentage of blood vessel area versus the whole area under the stereomicroscopic field (He et al., 2014). The areas occupied by the blood vessels were also quantified. Some YSMs were also embedded in paraffin wax, serially sectioned at 5 μm (Leica RM2126RT, Germany) and stained with hematoxylin & eosiin (H&E). The rest of the YSMs were harvested for RNA isolation.

Cell viability assay. The viability of human umbilical vein endothelial cells (HUVECs) was assessed by MTT assay as described previously (Cheng et al., 2012). The absorbance values were determined at 570 nm on a BIO-RAD Microplate Reader (Model 450, BIO-RAD, Hercules, California). Cell viability was indirectly established by the ratio of the absorbance value of Dex-treated cells relative to the control. The samples were analyzed in triplicates.

Cell colony formation assay. HUVECs were used in colony formation assay as previously described (Tsang et al., 2013). In brief, 500 HUVECs were plated onto 25-cm² culture dishes and maintained in culture medium containing different concentration of Dex or 0.1% DMSO, in a CO2 incubator for 7 days. The cells were fixed with 4% paraformaldehyde (PFA) for 20 min and stained with 0.5% crystal violet (Amresco, Solon, Ohio) for 1 h at room temperature. The total number of colonies in each treatment was counted. The experiments were repeated independently thrice.

Immunofluorescent staining and F-Actin staining. The extent of cell proliferation in HUVECs and MC3T3-E1 cells were examined. These cultures were incubated with antibody (ph3, 1:400, Santa Cruz Biotechnology, Dallas, Texas) or rabbit polyclonal PCNA (1:100, Santa Cruz Biotechnology) primary antibodies at 4°C overnight. Then Alexa Fluor 555 anti-rabbit IgG (1:1000, Invitrogen, California) secondary antibody was used for visualizing the primary antibody. For F-Actin detection, cultured cells were stained using phallolidin-Alexa-Fluor 488 (1:200, Invitrogen) at room temperature for 2 h. All the cells were counterstained with DAPI (1:1000, Invitrogen) at room temperature for 1 h. Cell proliferation quantitation was evaluated as a percentage of pH3+ HUVECs or PCNA+ MC3T3-E1 cells relative to their corresponding cells in control groups.

Scratch wound migration assay. A “scratch wound” was created by scraping a monolayer culture of MC3T3-E1 cells using a sterile 100 μl pipette as described previously (Cheng et al., 2012). The “wounded” cultures were then incubated in DMEM plus 10% FBS for 24–48 h. At selected time intervals, MC3T3-E1 cells that have migrated into the wound area were photographed using an inverted microscope, and the length of the wound gap was measured using IPP software. The assays were performed thrice using triplicate culture wells.

phalanx culture on CAM. We used methods as previously reported (Smith et al., 2013). In brief, a shell window was opened in the lateral sides of fertilized eggs incubated for 7.5 days. Then 17-day-old chick embryonic phalanges were grafted onto the CAM and 100 μl of 0.1% DMSO or 10⁻⁶ M Dex was added into the site confined by a silicon ring. The transplanted phalanges were incubated at 37°C for 7 days. The samples were photographed using a Canon Powershot SX130 IS digital camera. Some of the phalanges with accompanying CAM and blood vessels within the rings were harvested and fixed in 4% PFA. The both of the bone and the areas occupied by blood vessels in the CAM were photographed by stereomicroscope and quantified using the Image Pro-Plus 5.0 (IPP, Media Cybernetics) software (Cheng et al., 2014; He et al., 2014). The phalanges were also decalcified in 10% EDTA solution for 2 days at 4°C and embedded in paraffin. The samples were serially sectioned at 5 μm thickness on a microtome. Longitudinal sections of these bones were produced and further stained with H&E for histological observations. The rest of the phalanges and CAMs were harvested for RNA isolation.

Mineralization of MC3T3-E1 cultures. MC3T3-E1 cells (a mouse osteoblastic cell line) were suspended in DMEM supplemented with 10% FBS at a density of 2.0 × 10⁶/ml. 10 μl of the cell suspension was placed in each of a 6-well tissue culture plate (Costar, Bethesda). After 1 h pre-incubated at 37°C, all the wells were flooded with 500 μl of culture medium. Various concentrations of Dex or 0.1% DMSO were then introduced on the second day of plating. Following treatment with different concentration of Dex for 10 days, the cultures were fixed in 95% ethanol for 20 min, and then stained with 2% alizarin red dye (pH 4.2) for 30 min at room temperature to demonstrate the calcium deposits.

Semi-quantitative RT-PCR. Total RNA was extracted from the cells and tissues using a Trizol kit (Invitrogen). First-strand cDNA was synthesized to a final volume of 25 μl using a SuperScript RII first-strand kit (Invitrogen). Following reverse transcription, PCR amplification of the cDNA was performed using chck specific primers. The primers sequences are provided in Supplementary Figure 1. The PCR reactions were performed in a Bio-Rad S1000TM Thermal cycler (BIO-RAD) as described previously (Lu et al., 2014). The resolved PCR products were visualized using a transilluminator (SYNGENE, UK) and photographs were captured using a computer-assisted gel documentation system (SYNGENE). The intensity of the fluorescently stained bands was measured and normalized using the IPP software.

Data analysis. The data produced were presented as mean ± standard error (SE). ANOVA tests were performed on the results using a Graphpad Prism 5 software package (Graphpad Software, California). Samples were considered to be significantly different when P < .05.

RESULTS

Dex Exposure Accelerates Endochondral Ossification During Embryo Development

We have previously reported the effect of Dex on osteogenesis and how it accelerated bone mineralization (Cheng et al., 2014). To further investigate this phenotype, 1.5-day-old chick embryos were exposed to 10⁻⁶–10⁻⁴ M Dex or either 0.1% DMSO (control) for 7.5 days. The development of their long bone (including humerus, radius, and femur) was then determined using alcian blue and alizarin red double staining (Figs. 1A–L). We observed alizarin red positive staining in all of the humerus, radius, and femur and the intensity of staining increased in a Dex dose-dependent manner. The length of the humerus stained with alizarin red was measured between control and Dex treatments and statistical analyzed (control: 709.1 ± 8.92 μm; 10⁻⁶ Dex: 719.3 ± 16.41 μm; 10⁻⁴ Dex: 736.6 ± 13.54 μm; 10⁻³ Dex: 734.8 ± 11.75 μm; n = 6 for each group) (Figure 1M). For the radius the extent of staining was control: 729.6 ± 7.925 μm;
FIG. 1. Dex exposure during gastrulation accelerates long bone mineralization. Early chick embryos (Day 1.5) were treated with $10^{-6}$–$10^{-7}$ M Dex for 7.5 days and then the skeletal structures were stained with alcian blue and alizarin red dyes. A–L: Representative appearance of 9-day chick embryos’ humerus (A–D), radius (E–H), and femur (I–L) treated with 0.1% DMSO (control) or $10^{-6}$–$10^{-7}$ M Dex. M–O: Bar charts comparing the length of alizarin red-stained humerus (M), radius (N), and femur (O) between control and Dex-treated embryos. **$P < .01$ compared with control. Scale bars = 500 μm in A–L.

FIG. 2. Dex exposure does not influence phalange mineralization in vitro. Phalanges were isolated from 17-day-chick embryos and cultured in vitro for 8 days in absence or presence of $10^{-6}$–$10^{-4}$ M Dex. A–F: Representative appearance of phalange cultures in vitro, at day 0. A–F: Representative appearance of phalange cultures in vitro, at day 8. G: Bar chart comparing the relative growth rate of the cultured phalanges between control and Dex-treated groups. H: Bar chart comparing the extent of mineralization in control and Dex-treated phalanges. **$P < .01$ compared with control. Scale bars = 1 mm in A–F and A–F.
FIG. 3. Dex exposure promotes angiogenesis in YSM. YSM assay was used to determine the effect of Dex exposure on angiogenesis. The colored ink marks on the silicon rings define the leading edges of the developing blood vessel plexus in YSM of 2.5-day-chick embryos. A–D, Representative appearance of the blood vessel plexus 0 (A), 12 (B), 24 (C), and 36 (D) hours in the control group. A’–D’, Representative appearance of the blood vessel plexus 0 (A’), 12 (B’), 24 (C’), and 36 (D’) hours in Dex-treated group. E–E’, Representative transverse sections of YSM from D and D’, respectively. B’–D’, Bar chart comparing the BVD from 12 to 36 h between control and Dex-treated groups. G–H, Semi-quantitative RT-PCR and bar chart showing the extent of VEGF-R2, FGF2, and Ang-1 expression in YSM following Dex treatment. *P < .05 compared with control, **P < .01 compared with control. Scale bars = 1 mm in A–D/A’–D’ and 100 μm in E–E’.

10^{-9} \text{Dex}: 735.2 \pm 6.190 \mu m; 10^{-8} \text{Dex}: 736.5 \pm 7.627 \mu m; 10^{-7} \text{Dex}: 786.2 \pm 10.27 \mu m; n = 6 for each group) (Figure 1N); For the femur, the extent of staining was control: 981.3 \pm 11.93 \mu m; 10^{-9} \text{Dex}: 1134 \pm 76.58 \mu m, 10^{-8} \text{Dex}: 1159 \pm 36.34 \mu m, 10^{-7} \text{Dex}: 1187 \pm 48.60 \mu m; n = 6 for each group) (Figure 1O). These results imply that Dex exposure during embryogenesis significantly accelerates bone mineralization.

Dex Treatment Does Not Affect Ossification in Phalange Cultures

We exposed isolated phalanges to 10^{-8}–10^{-4} M Dex for 8 days in vitro to quantitatively evaluate the effect of Dex on ossification during long bone development (Figs. 2A–F and A’–F’). We observed a distinct enhancement of cartilage growth but the relative lengths of the cultured phalanges did not significantly increase after Dex treatments exposing to Dex for 1 or 8 days in comparison to control (1-day-control: 0.87 \pm 0.06 \mu m; 10^{-8} \text{Dex}: 0.91 \pm 0.05 \mu m, 10^{-7} \text{Dex}: 0.99 \pm 0.46 \mu m, 10^{-6} \text{Dex}: 0.99 \pm 0.02 \mu m, 10^{-5} \text{Dex}: 0.89 \pm 0.05 \mu m, 10^{-4} \text{Dex}: 0.91 \pm 0.11 \mu m; 8-day-control: 1.15 \pm 0.05 \mu m; 10^{-8} \text{Dex}: 1.17 \pm 0.26 \mu m, 10^{-7} \text{Dex}: 1.07 \pm 0.09 \mu m, 10^{-6} \text{Dex}: 1.09 \pm 0.10 \mu m, 10^{-5} \text{Dex}: 0.96 \pm 0.08 \mu m, 10^{-4} \text{Dex}: 0.93 \pm 0.09 \mu m; n = 6 for each group) (Figure 2G). We also examined the extent of mineralization in the cultured phalanges exposed to Dex. Dex treatment did not significantly increase mineralization, as determined by the length of the phalanges stained with alizarin red (1-day-control: 63.39 \pm 12.06 \mu m; 10^{-8} \text{Dex}: 62.81 \pm 12.73 \mu m, 10^{-7} \text{Dex}: 62.52 \pm 21.27 \mu m, 10^{-6} \text{Dex}: 58.44 \pm 16.19 \mu m, 10^{-5} \text{Dex}: 58.86 \pm 10.01 \mu m, 10^{-4} \text{Dex}: 51.79 \pm 8.97 \mu m; 8-day-control: 68.01 \pm 5.21; 10^{-8} \text{Dex}: 81.57 \pm 13.98, 10^{-7} \text{Dex}: 84.87 \pm 18.97, 10^{-6} \text{Dex}: 83.17 \pm 23.08, 10^{-5} \text{Dex}: 74.5 \pm 15.51, 10^{-4} \text{Dex}: 63.94 \pm 12.17; n = 6 for each group) (Figure 2H). The results show a distinct difference between in vitro and ex viv o bone response to Dex.

Dex Exposure Promotes Angiogenesis in Chick YSM

We investigated the effect of Dex exposure on angiogenesis using the YSM angiogenesis model (Figure 3) (Cheng et al., 2015). The Dex or DMSO was administrated into the silicon rings as shown in Figures 3A–D’ . The YSM blood vessel plexus normally
grow and expand from left to right of silicon rings. These rings are also useful in retaining the Dex and DMSO in one place on YSM. The starting point of the YSM blood vessels is marked with red/black inks on the silicon rings and is kept constant in all replicates (Figs. 3A–D and A’–D’). We determined that 10⁻⁷M Dex treatment significantly increased the speed of expansion of the blood vessel plexus compared with the control. This is indicated by the leading edges of Dex-treated blood vessel plexus reaching the rings after 12 h incubation, and beyond the rings after 24 h (Figs. 3A–D and A’–D’). The BVD was significantly increased in 10⁻⁷M Dex-treated vessels after 12 h incubation (control: 0.38 ± 0.02; 10⁻⁶ M Dex: 0.44 ± 0.01; n = 7 for each group), after 24 h incubation (control: 0.42 ± 0.03; 10⁻⁶ M Dex: 0.47 ± 0.02; n = 7 for each group), and after 36 h incubation (control: 0.45 ± 0.04; 10⁻⁶ M Dex: 0.53 ± 0.01; n = 7 for each group) (Figs. 3B’–D’ and F). Transverse histological sections were produced from the experimental YSM. The area of transverse sections occupied by blood vessels were determined and revealed that Dex exposure significantly increased the amount of blood vessels (control: 2.17 ± 0.16; Dex: 2.38 ± 0.21; n = 7 for each group) (**P < .01 compared with control). These results imply that Dex exposure enhanced angiogenesis in chick YSM.

Next, we employed HUVECs to test the effects of Dex on endothelial cell proliferation and migration. First, MTT analysis showed that at concentrations >10⁻⁷ M Dex could significantly increase HUVECs proliferation (control: 1.0 ± 0.01%; 10⁻⁸ M Dex: 1.03 ± 0.01%, 10⁻⁷ M Dex: 1.15 ± 0.01%, 10⁻⁶ M Dex: 1.23 ± 0.02%, 10⁻⁵ M Dex: 1.14 ± 0.01%, 10⁻⁴ M Dex: 1.14 ± 0.01%; n = 6 for each group) (Figure 4A). This enhancement of HUVECs proliferation was also reflected by an increase in the number of HUVECs clones after Dex treatment, comparison with the control (control: 394.3 ± 53.54; 10⁻⁸ M Dex: 377 ± 70.19, 10⁻⁷ M Dex: 607.7 ± 78.14, 10⁻⁶ M Dex: 746.7 ± 30.44, 10⁻⁵ M Dex: 805 ± 60.1, 10⁻⁴ M Dex: 540 ± 27.4; n = 3 for each group) (Figs. 4B–H). pH3 immunofluorescent staining was performed on HUVECs cultured in DMSO (K) and 10⁻⁶ M Dex (L) for 24 h to demonstrate extent of cell proliferation. K’–L’, Merge images of K–L and DAPI staining. M, Bar chart comparing the number of pH3⁺ HUVECs in control and Dex cultures. N–O, Scratch wound cell migration assay showing the appearance of control and 10⁻⁴ M Dex cultures at 0 (N–O), 24 (N–O’), and 48 (N–O”–O’) hours. P, Bar chart comparing the size of the gap in the wound site (distance) as HUVECs migrate into the wound at different time intervals in DMSO and Dex (10⁻⁶ M) treated HUVECs cultures. *P < .05 compared with control, **P < .01 compared with control. Scale bars = 10 mm in B-G, 100 µm in K’–L’–K”–L”–K”’–L”’–K”’’–L”’’, and 200 µm in N–O/N’–O’/N”–O”/N”’–O”’.’

FIG. 4. Dex exposure increases HUVECs proliferation and migration. HUVECs were used to determine the effect of Dex on endothelial cell proliferation and migration. A, Bar chart showing HUVECs viability after DMSO (control) and Dex (10⁻⁸–10⁻⁴ M) treatment for 24 h. B–G, Images showing the extent of colony formation in DMSO and Dex (10⁻⁶–10⁻⁴ M) treated HUVECs for 24 h. H, Bar chart comparing the colonies numbers between DMSO and Dex (10⁻⁶–10⁻⁴ M) treated HUVECs for 24 h. K–L, pH3 immunofluorescent staining was performed on HUVECs cultured in DMSO (K) and 10⁻⁶ M Dex (L) for 24 h to demonstrate extent of cell proliferation. K’–L’, Merge images of K–L and DAPI staining. M, Bar chart comparing the number of pH3⁺ HUVECs in control and Dex cultures. N–O, Scratch wound cell migration assay showing the appearance of control and 10⁻⁴ M Dex cultures at 0 (N–O), 24 (N–O’), and 48 (N–O”–O’) hours. P, Bar chart comparing the size of the gap in the wound site (distance) as HUVECs migrate into the wound at different time intervals in DMSO and Dex (10⁻⁶ M) treated HUVECs cultures. *P < .05 compared with control, **P < .01 compared with control.
Dex Promotes Angiogenesis in Phalanges Cultured on CAM
Phalanges were isolated from 17-day-old chick embryos and grafted onto 7.5-day chick embryonic CAM (Smith et al., 2013). These phalanges were incubated in the presence DMSO (control) or 10^{-6}M Dex for 7 days. We observed that the phalanges in both groups grow well on CAM (Figs. 5A–B). The blood vessels radiating from the chick embryos were distributed around the grafted phalanges, which indicates that the cultured phalanges have acquired a blood supply through de novo angiogenesis (Figs. 5A–B and A’–B’). We determined that the lengths of cultured phalanges treated with Dex were significantly shorter than control phalanges (control: 4304 ± 488.7 μm; 10^{-6} Dex: 3534 ± 136.4 μm; n = 4 for each group) (Figure 5C). In addition, the expression of HIF-1α and Ang-1 were determined to be up-regulated following Dex treatment (VEGF-R2 control: 1.7 ± 0.10, Dex: 1.7 ± 0.10; FGF2 control: 1.3 ± 0.07, Dex: 1.4 ± 0.10; HIF-1α control: 1.0 ± 0.15, Dex: 1.6 ± 0.20; Ang-1 control: 1.0 ± 0.07, Dex: 1.4 ± 0.10) (Figure 5E). Moreover, the expression of osteogenesis-related genes, Runx2 (control: 1.0 ± 0.05, Dex: 1.2 ± 0.06; n = 3 for each group) and Colla1 (control: 1.0 ± 0.04, Dex: 4.7 ± 0.48; n = 3 for each group) was up-regulated in phalanges cultured in the presence of Dex (Figure 6E).

Phalanges were incubated in the presence DMSO (control) or 10^{-6}M Dex. The results showed that Dex exposure significantly increased the number of pH3+ HUVECs in the cultures compared with the control (control: 0.017 ± 0.004; 10^{-6} Dex 0.030 ± 0.004; n = 5 for each group) (Figs. 4K–M and K’–L’). Lastly, our scratch wound assay showed that Dex exposure enhanced HUVECs migration and this was reflected in the extent of “wound” closure between the control and Dex-treated groups after 48 h incubation (0-h-control: 507.2 ± 40.94 μm; Dex: 517.5 ± 70.09 μm; 24-h-control: 367.7 ± 40.21 μm, Dex: 356.1 ± 84.72 μm; 48-h-control: 204.7 ± 23.69 μm, Dex: 145.8 ± 49.53 μm; n = 8 for each group) (Figs. 4N–P, N’–O’). These results indicate that Dex exposure could promote HUVECs proliferation and migration.

Staining was performed on the HUVECs treated with DMSO or 10^{-6}M Dex. The results showed that Dex exposure significantly increased the number of pH3+ HUVECs in the cultures compared with the control (control: 0.017 ± 0.004; 10^{-6} Dex 0.030 ± 0.004; n = 5 for each group) (Figs. 4K–M and K’–L’). Lastly, our scratch wound assay showed that Dex exposure enhanced HUVECs migration and this was reflected in the extent of “wound” closure between the control and Dex-treated groups after 48 h incubation (0-h-control: 507.2 ± 40.94 μm; Dex: 517.5 ± 70.09 μm; 24-h-control: 367.7 ± 40.21 μm, Dex: 356.1 ± 84.72 μm; 48-h-control: 204.7 ± 23.69 μm, Dex: 145.8 ± 49.53 μm; n = 8 for each group) (Figs. 4N–P, N’–O’). These results indicate that Dex exposure could promote HUVECs proliferation and migration.

**FIG. 5.** Dex promotes angiogenesis in CAM surrounding the phalanges. Phalanges (isolated from 17-day-old chick embryos) were grafted onto 7.5-day chick embryo CAM, and cultured for 7 days. A–B, The representative appearance of control (A) and 10^{-6}M Dex (B) phalanges cultured on CAM for 7 days. A’–B’, Higher magnification of black square labeled region in A and B, respectively. A’–B’, Higher magnification of region highlighted by the black squares in A’ and B’, respectively. C, Bar chart comparing the lengths of the cultured phalanges on CAM between control and Dex groups. D, Bar chart comparing the blood vessel areas adjacent to the cultured phalanges within the silicon ring on CAM between control and Dex groups. E–F, Semi-quantitative RT-PCR and bar chart showing the extent of VEGF-R2, FGF2, HIF-1α, and Ang-1 expressions in DMSO and Dex-cultured CAM and phalanges within the silicon rings. *P < 0.05 compared with control. Scale bars = 2 mm in A–B, 1 mm in A’–B’, and 500 μm in A’–B’.

Longitudinal sections were produced from the phalange cultures (Figs. 6A–B). We observed that the number of osteoblasts was dramatically reduced in the Dex-treated phalange compared with the control (control: 125.5 ± 10.79; 10^{-6} Dex: 77.5 ± 11.73; n = 4 for each group) (Figs. 6A’–B’, A”–B’’, and O). In contrast, the number of osteocytes was significantly increased in the Dex-treated phalange compared with control (41.99 ± 8.74; 10^{-6} Dex: 61.01 ± 9.51; n = 4 for each group) (Figs. 6A’–B’, A”–B’’, and D). Moreover, the expression of osteogenesis-related genes, Runx2 (control: 1.0 ± 0.05, Dex: 1.2 ± 0.06; n = 3 for each group) and Cola1 (control: 1.0 ± 0.04, Dex: 4.7 ± 0.48; n = 3 for each group) was up-regulated in phalanges cultured in the presence of Dex (Figure 6E).
Dex Inhibits Cell Proliferation and Enhances Mineralization in MC3T3-E1 Cultures

We found MC3T3-E1 cell viability was inhibited by Dex in a dose-dependent manner ($10^{-8}$ Dex: 0.94 ± 0.05, $10^{-7}$ Dex: 0.87 ± 0.02, $10^{-6}$ Dex: 0.87 ± 0.04, $10^{-5}$ Dex: 0.89 ± 0.03, $10^{-4}$ Dex: 0.87 ± 0.02; n = 5 for each group) in comparison to control (1.0 ± 0.03) (Figure 7G). This inhibitory effect of Dex was further confirmed by PCNA immunofluorescent staining on Dex-treated MC3T3-E1 cells (control: 0.13 ± 0.02; $10^{-8}$ Dex: 0.12 ± 0.01, $10^{-7}$ Dex: 0.11 ± 0.01, $10^{-6}$ Dex: 0.10 ± 0.01, $10^{-5}$ Dex: 0.10 ± 0.01, $10^{-4}$ Dex: 0.08 ± 0.01; n = 5 for each group) (Figs. 7A–F and H). Actin polymerization was determined by phalloidin-Alexa-Fluor 488-labeled antibody and fluorescence microscopy. We found that Dex significantly weakened the cytoskeletal organization too (Figs. 7I–N). The cultures were stained with alizarin red dye and revealed that Dex significantly accelerated the mineralization MC3T3-E1 cultures as compared with the control (control: 1124 ± 122.5, $10^{-8}$ Dex: 1753 ± 170.7, $10^{-7}$ Dex: 1756 ± 50.17, $10^{-6}$ Dex: 1862 ± 69.7, $10^{-5}$ Dex: 1865 ± 72.48, $10^{-4}$ Dex: 1823 ± 82.46; n = 4 for each group) (Figs. 7O–T, U).

DISCUSSION

GCs are the steroid hormones capable of binding with GC receptors found in many cells in the human body. It is almost indispensable for maintaining normal physiological functions such as growth and metabolism. In addition, GCs play an important role in regulating osteogenesis and maintaining homeostasis in cartilage and bone (Olney, 2009; Silbermann and Maor, 1978; Silbermann et al., 1977). GCs have been extensively used in clinics to suppress inflammation and modulate immunity in chronic inflammatory, asthma, rheumatoid arthritis, and autoimmune diseases (Barnes, 1998). A wider range of Dex application has been hampered because of its severe side effects, despite its efficiency in alleviating disease symptoms. These side effects include inhibiting endochondral bone growth in fetus and adolescents, and accelerating osteoporosis in adults (Raff, 2004; Warner, 1995). Therefore, pregnant women inflicted with severe diseases that need GCs therapy could only be given GCs for a relatively short period (Ahmed et al., 1999). Nevertheless, there is increasing evidence that a single usage of GCs is sufficient to cause irreversible malformations during embryo development. In this context, the more we understand about the influence of GCs on osteogenesis during embryogenesis, the more precautions we can take to avoid the damaging side effects of GCs. In our previous study, we have investigated the whole influence of GCs application on osteogenesis (Cheng et al., 2014). Here, we focused on the effect of GCs on mineralization and angiogenesis during bone development.

During long bone formation, endochondral ossification is composed of the following 2 crucial steps, the formation of cartilage template and the subsequent replacement of the
template with vasculature, osteoblasts, osteoclasts, and bone matrix. Presently, we have found that the mineralization process was accelerated by exposure to Dex during long bone development. This was clearly demonstrated by the extent of calcification of the humerus, radius, and femur. The accelerated mineralization induced by Dex exposure was presented in a dose-dependent manner \textit{in vivo}. We have already reported that Dex treatment inhibited the length of developing long bones (Cheng et al. 2014) and this study suggests that this was most likely to be attributed to accelerated bone mineralization. The results are schematically summarized in Figure 8.

First, we cultured phalanges \textit{in vitro} in absence and presence of Dex. To our surprise, we did not find a striking increase in mineralization of the Dex-treated phalanges as seen \textit{in vivo}. This difference, we reasoned, was attributed to angiogenesis which is absent \textit{in vitro}. Hence, we used a chick embryonic YSM model to investigate whether or not Dex exposure affected angiogenesis (Cheng et al. 2015). The results indicated that Dex exposure enhanced angiogenesis in a dose-dependent manner. This observation was further supported by semi-quantitative RT-PCR which showed an increase in angiogenesis-related gene expression from Dex-treated YSM. To elucidate how Dex affected angiogenesis, we treated HUVECs with different concentrations of Dex and also performed cell scratch wound assay. The results revealed that Dex exposure enhanced HUVECs proliferation and migration. These \textit{in vivo} and \textit{in vitro} angiogenesis experiments suggest that Dex might stimulate
angio genesis during osteogenesis which in turn increases bone mineralization. To confirm this hypothesis, we cultured phalanges on chick CAM in the presence of DMSO (control) or Dex. The results revealed that the blood vessel density surrounding the cultured phalanges was significantly increased by Dex treatment and also expressions of angiogenesis-related genes (VEGF-R2, FGF2, HIF-1α, and Ang-1). VEGF, Ang-1, and FGF-2 have been reported to exert various bioactivities on angiogenesis during osteoblasts differentiation that include stimulation of cell growth, migration, wound healing, tissue repair, differentiation, and morphogenesis (Kigami et al., 2013; Olives-Navarrete et al., 2013). In addition, all of above factors are known to play critical roles in bone growth and development (Bentovim et al., 2012; Mansukhani et al., 2000; Wallner et al., 2015). These findings seems to confirm our hypothesis.

We further explore the causes of Dex-induced accelerated mineralization. We estimated the number of osteoblasts in cultured phalanges and determined that Dex treatment dramatically reduced the number of osteoblasts while increased the number of osteocytes. In this regards, it has been reported before that GCs exposure leads to decrease generation of osteoblasts and osteocytes, while the lifespan of osteoclasts was prolonged (Canalis, 2005). It seemed that the inhibition of osteoblast activity is typically attended by parallel changes in the osteoclasts. However, there was no notable change on osteoclasts in our observation. We speculate that bone turnover and absorption is comparatively less active during embryonic period but after birth (when the skeleton bears the body weight) the number of osteoclasts increases. Moreover, Dex disrupted the cytoskeleton of MC3T3-E1, which directly affected the cell’s stability and may indirectly exert an effect on the cell’s differentiation. The terminal differentiation of osteoblasts into osteocytes might be prematurely stimulated by Dex exposure. In support, we found Dex enhanced Runx-2 and Col10α1 expression in cultured phalanges. This was further confirmed by Dex exposure inhibiting MC3T3-E1 proliferation. It has been reported that high doses of GCs treatment in mice could increase apoptosis in osteocytes (O’Brien et al., 2004). Nevertheless, other studies demonstrated that GCs induces autophagy to preserve osteocyte viability (Xia et al., 2010). It follows that bone loss caused by GCs does not occur to the same extent in different studies, which is partly due to the multifunctional nature of GCs. Furthermore, increased mineralization seen in our MC3T3-E1 cells cultured in the presence of Dex further support our above hypothesis.

In summary, we have utilized a combination of in vivo and in vitro experimental approaches to demonstrate Dex exposure promoted angiogenesis during long bone formation. It was achieved through the stimulation of endothelial cell proliferation and migration. Dex exposure might also influence osteoblasts differentiation into osteocytes by inhibiting the proliferation and viability of osteoblasts.

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**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

**REFERENCES**


