

[Original]

## Control of Survival by Hypoxia-Inducing Factors through Glucose Transporters, Monocarboxylate Transporters and Autophagy in Chondrocytes of Developing Mouse Tibias

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**Key words :** Hypoxia-inducing factors, Glucose transporters, Monocarboxylate transporters, Autophagy, Epiphyseal chondrocytes

### Abstract

The mechanism by which chondrocytes survive in an avascular environment remains unknown. Hypoxia induces and regulates the expression of many genes associated with proliferation and growth arrest, survival and apoptosis, glucose transport and metabolism, and autophagy, via hypoxia-inducing factors (HIFs). In hypoxic milieu, glucose transporters (GLUTs) and monocarboxylate transporters (MCTs) are involved in rapid transport of energy fuel such as monosaccharides, pyruvate, lactate and ketone bodies across the plasma membrane. HIFs are also involved in the regulation of autophagy during the hypertrophy and apoptosis of chondrocytes. Immunohistochemical localization of HIFs, GLUTs, MCTs and autophagy processing marker LC3B was examined in epiphyseal chondrocytes of developing mouse tibias. Glycogen accumulation was seen in prehypertrophic and hypertrophic chondrocytes of 1- and 4-week-old cartilages and a few hypertrophic chondrocytes of 8-week-old growth plate. Intense HIF-1 $\alpha$  immunoreaction was seen in prehypertrophic and hypertrophic chondrocytes of 1-, 4- and 8-week-old cartilages, but HIF-2 $\alpha$  was depressed in hypertrophic chondrocytes of 8-week-old cartilage. Immunoreactivities of GLUT1, GLUT5 and GLUT9 were moderate

and faint in prehypertrophic and hypertrophic chondrocytes of 1-week-old cartilage and subsequently decreased. However, GLUT3 immunoreaction continued to be intense. MCT1, MCT2 and MCT4 were intense and moderate in prehypertrophic and hypertrophic chondrocytes of 1-week-old cartilage, whereas MCT2 was moderate even in 8-week-old cartilage. LC3B was distributed in the zones from the proliferating to hypertrophic chondrocytes of 1- and 4-week-old cartilages. In contrast, LC3B was mainly confined to hypertrophic chondrocytes of 8-week-old growth plate. These results indicated co-localization of HIF-1 $\alpha$ , GLUT3, MCT2 and LC3B in prehypertrophic and hypertrophic chondrocytes in epiphyseal cartilage and growth plates. However, HIF-2 $\alpha$  immunoreaction disappeared only in 8-week-old growth plate. At this stage, HIF-1 $\alpha$ , which is accelerated by suppression of HIF-2 $\alpha$ , may induce metabolic change from glycogen accumulation to immediate consumption and autophagy without sufficient hypertrophy. We suggest that survival of chondrocytes may be controlled by activation of HIF-1 $\alpha$  and HIF-2 $\alpha$  through GLUTs, MCTs and autophagy in epiphyseal cartilage and growth plate of mouse tibias.

### INTRODUCTION

In endochondral ossification, the cartilaginous temple is replaced by bone in diaphysis and both epiphyses (epiphy-

seal cartilage and growth plate) are responsible for longitudinal bone growth. However, the cartilaginous tissue is a virtually avascular tissue, and the chondrocytes consequently reside in hypoxic conditions (Schipani, 2005 ; Schipani et

al., 2001). Under severe environments, chondrocytes survive and contribute to bone growth and formation in endochondral ossification.

Under hypoxia, hypoxia-inducing factor (HIF)-1 is necessary for a metabolic shift from the oxidative phosphorylation pathway in the mitochondria to the oxygen-independent glycolytic pathway in the cytoplasm (Seagroves et al., 2001). Monosaccharides, an important energy source in hypoxic milieu, are transported to the cytoplasm across the plasma membranes of mammalian cells, and then mediated by members of the facilitative Na<sup>+</sup>-independent sugar transporter (GLUT/SLC2A) and Na<sup>+</sup>-dependent glucose co-transporter (SGLT/SLC5A) families (Wood & Trayhurn, 2003). In the epiphyseal cartilage of postnatal rat tibias, glucose transporter (GLUT)1 immunoreactivity has been found in pre-hypertrophic chondrocytes, whereas GLUT2 and GLUT3 immunoreactivity is weak in all chondrocytes, and intense GLUT4 and GLUT5 immunoreactivity is localized in hypertrophic chondrocytes (Ohara et al., 2001). In addition, monocarboxylate transporters (MCTs) are also involved in rapid transport of pyruvate, lactate and ketone bodies across the plasma membrane, which are available for carbohydrate, fat and amino acid metabolism inside cells (Halestrap, 2012; Halestrap & Meredith, 2004; Halestrap & Wilson, 2012). MCT1 plays a predominant role in influx or efflux of pyruvate, lactate and ketone bodies across the plasma membrane in a majority of tissues in all species. Under hypoxic conditions, however, most cells export lactic acid to reduce intracellular acidification. MCT4 is expressed especially in cells that rely on glycolysis for production of respiratory fuel such as chondrocytes, white skeletal muscle fibers, astrocytes and white blood cells. Autophagy is a natural process by which dysfunctional organelles are degraded inside the cell through lysosomal action, and is particularly activated under severe conditions such as hypoxia and starvation. Furthermore, HIFs are crucially involved in the regulation of autophagy during the hypertrophy of chondrocytes in the growth plate of endochondral ossification (Bohensky et al., 2009; 2010; Stewart et al., 2006; Srinivas et al., 2009a; Zhang et al., 2008). Thus, HIFs may be a key regulator for survival of chondrocytes through GLUTs and MCTs expression, in addition to regulation of autophagy.

Unfortunately, the mechanism by which chondrocytes survive in an avascular environment remains unknown. In the present study, we examined the immunohistochemical local-

ization of HIFs, GLUTs, MCTs and autophagy processing marker LC3B in the chondrocytes of epiphyseal cartilage and growth plate.

## MATERIALS AND METHODS

### Ethical approval

The experimental protocol used was approved by the Animal Ethics and Research Committee of the Health Sciences University of Hokkaido, and the study was conducted in accordance with our institutional Regulations for the Care and Use of Laboratory Animals (Approval Number : No. 40, March 18, 2011).

### Tissue preparation

Each of three ddY strain mice at 1, 4 and 8 weeks of age was perfused by 4% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.2), and the tibias were dissected for tissue preparation of paraffin embedded and frozen sections. The specimens were decalcified with 4.13% EDTA for 3–4 weeks. After decalcification, the proximal half part of the bone was cut and embedded in paraffin. The sections were cut along a longitudinal axis of the long bone and stained with hematoxylin and eosin (H-E) and periodic acid–Schiff (PAS) reaction as well as through immunoperoxidase histochemistry. In a part of the tibias, un-decalcified frozen sections were conducted with von Kossa's reaction and by immunofluorescence histochemistry of autophagy marker.

### Periodic Acid–Schiff (PAS) reaction and von Kossa's reaction

Frozen sections were re-hydrated and then treated with 0.5% periodic acid for 5 min. The specimens were reacted with Schiff's reagent for 15 min, rinsed in 0.5% sodium hydrogen sulfate solution (3 x 3 min), and washed in running water for 5 min. The sections were then counterstained with Delafield's hematoxylin, washed in running water for 15 min, dehydrated in ethanol, cleared in xylene, and mounted in Eukitt (O. Kindler, Freiburg, Germany). As negative controls for glycogen store in chondrocytes, de-paraffinized and re-hydrated sections were treated by salivary digestion at 37°C for 30 min.

To explore the calcification of epiphysis, un-decalcified frozen sections were processed using von Kossa's reaction. The sections were incubated with 5% silver nitrate for 60

min under a fluorescent lamp. Thereafter, silver deposits in the specimens were fixed with 5% sodium thiosulfate for 3 min. After rinsing with tap water, the sections were then counterstained with methyl green, washed in running water for 15 min, dehydrated in ethanol, cleared in xylene, and mounted in Eukitt.

### Immunohistochemistry of HIFs, GLUTs and MCTs

Purified rabbit polyclonal antibodies against human HIF-1 $\alpha$  and mouse/human HIF-2 $\alpha$  were purchased from Upstate (Lake Placid, NY, USA) and Novus Biologicals (Littleton, CO, USA). An affinity-purified rabbit polyclonal antibody against rat GLUT1 was obtained from Abcam (Abcam, Tokyo, Japan). Affinity-purified goat anti-mouse GLUT3, mouse GLUT5, human GLUT9, mouse MCT1, mouse MCT 2 and human MCT4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Normal rabbit and goat sera were obtained from Cedarlane Laboratories (Hornby, British Columbia, Canada) and Biomedica (Foster City, CA, USA), respectively.

De-paraffinized sections were rinsed in 0.01 M phosphate-buffered saline (PBS, 3 x 10 min), followed by incubation in 0.01% hydroxyl oxygen/PBS for 30 min and then in cold PBS containing 10% bovine serum albumin (BSA) for 30 min. After washing with PBS (3 x 10 min), the sections were reacted with the above antibodies (diluted 1 : 50 in 0.1% BSA/PBS) overnight at 4°C, and then incubated with a Vectastain Elite ABC kit for rabbit and goat immunoglobulin (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's protocol. Immunoreactivity was visualized with 0.004% 3,3-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan) and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris-HCl (pH 7.6) for 10 min. After washing in distilled water, the sections were counterstained with methyl green. To confirm the specificity of the immunostaining, the primary antibodies were substituted with normal rabbit or goat serum at the same dilution.

### Immunofluorescence staining of LC3B

The frozen sections were stained with rabbit monoclonal anti-human LC3B antibody obtained from Novus Biologicals (Centennial, CO, USA). The frozen sections were rehydrated with PBS, treated with cold PBS containing 10% BSA for 30 min, and then incubated overnight with primary antibody in a dark humidified box at room temperature. Af-

ter rinsing three times for 10 min, the specimens were incubated with Alexa Fluor 488 conjugated F(ab')<sub>2</sub>-goat polyclonal anti-rabbit IgG (H+L) antibody (Invitrogen, USA) for 60 min at room temperature in a dark humidified box. After rinsing, the sections were mounted using Vectashield Hard-Set antifade mounting medium (Vector, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI) and observed at emission wavelength of 488 nm for the secondary antibody and 360 nm for DAPI. The sections were observed under a fluorescence microscope and the obtained images were overlaid for detection of LC3B-positive cells.

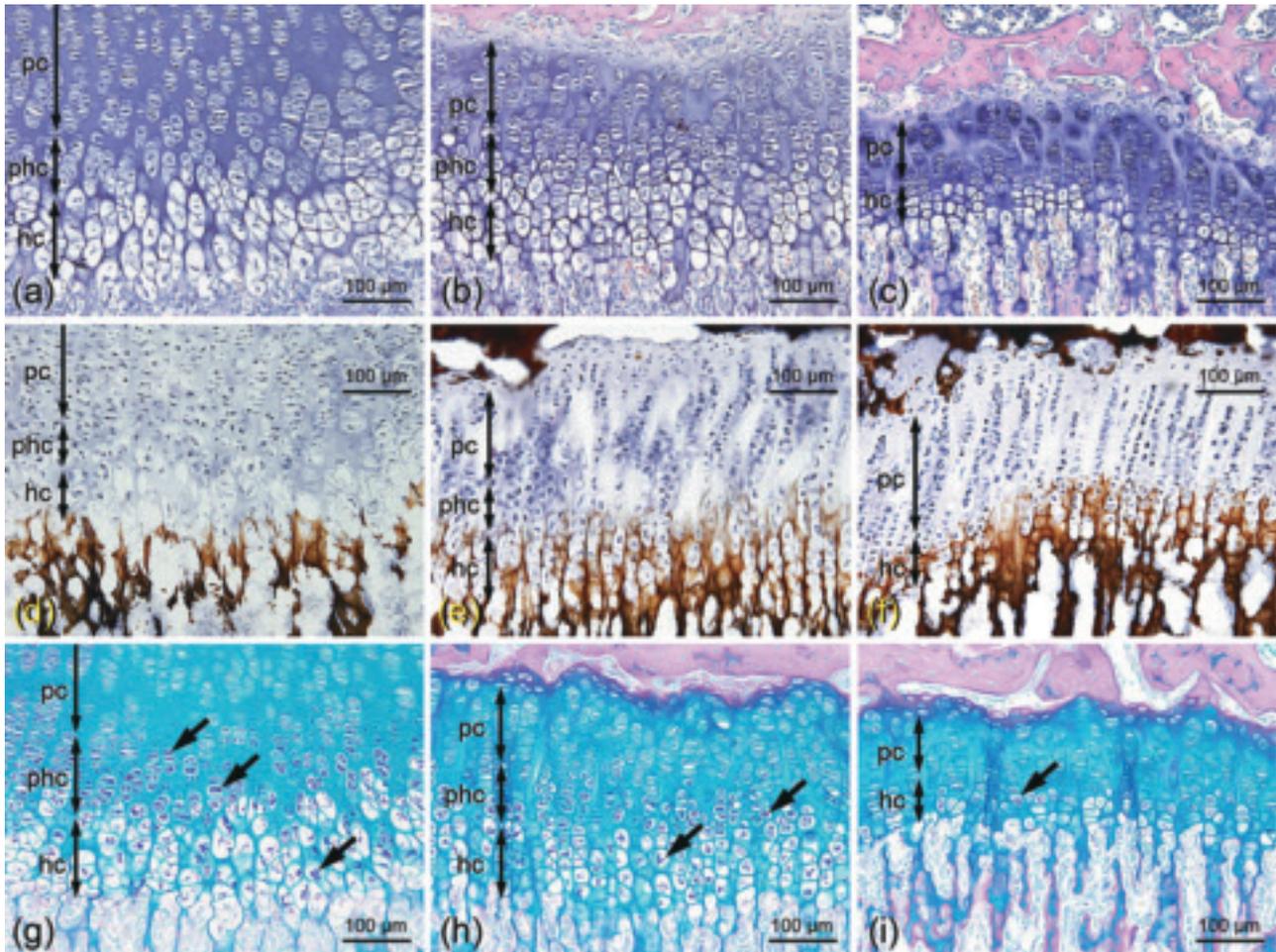
## RESULTS

### Histological features, calcium deposits, and glycogen accumulation

The epiphyseal cartilage of 1-week-old mouse tibias consisted of three zones of proliferating chondrocytes, prehypertrophic and hypertrophic chondrocytes (Figure 1a). At 4 weeks after birth, the zones of proliferating and prehypertrophic chondrocytes became narrow in the growth plate (Figure 1b). In 8-week-old mouse tibias, the growth plate was observed as narrow zones of proliferating and hypertrophic chondrocytes (Figure 1c). At this stage, prehypertrophic chondrocytes were not observed, and chondrocytes at the diaphyseal side were not fully hypertrophied in cell size. However, in this study, the cells were called hypertrophic chondrocytes because they immediately died. Calcium deposits indicated using von Kossa's reaction were seen in the primary bone trabecular but not in the matrix around hypertrophic chondrocytes in 1-week-old mice (Figure 1d). However, the deposits were clearly observed in the matrix around the prehypertrophic and hypertrophic chondrocytes at the growth plate of 4- and 8-week-old mice (Figure 1e, f). Glycogen accumulation presented by PAS reaction was predominantly intense in the defined zone of prehypertrophic chondrocytes in 1- and 4-week-old mice (Figure 1g, h). The reaction was observed in only a few hypertrophic chondrocytes in the growth plate of 8-week-old mice (Figure 1i).

### Immunolocalization of HIFs, GLUTs, MCTs and autophagy processing marker LC3B

In the epiphyseal cartilage of 1-week-old mouse tibias, immunointensity of HIF-1 $\alpha$  was moderate in the proliferating and intense in the prehypertrophic and hypertrophic



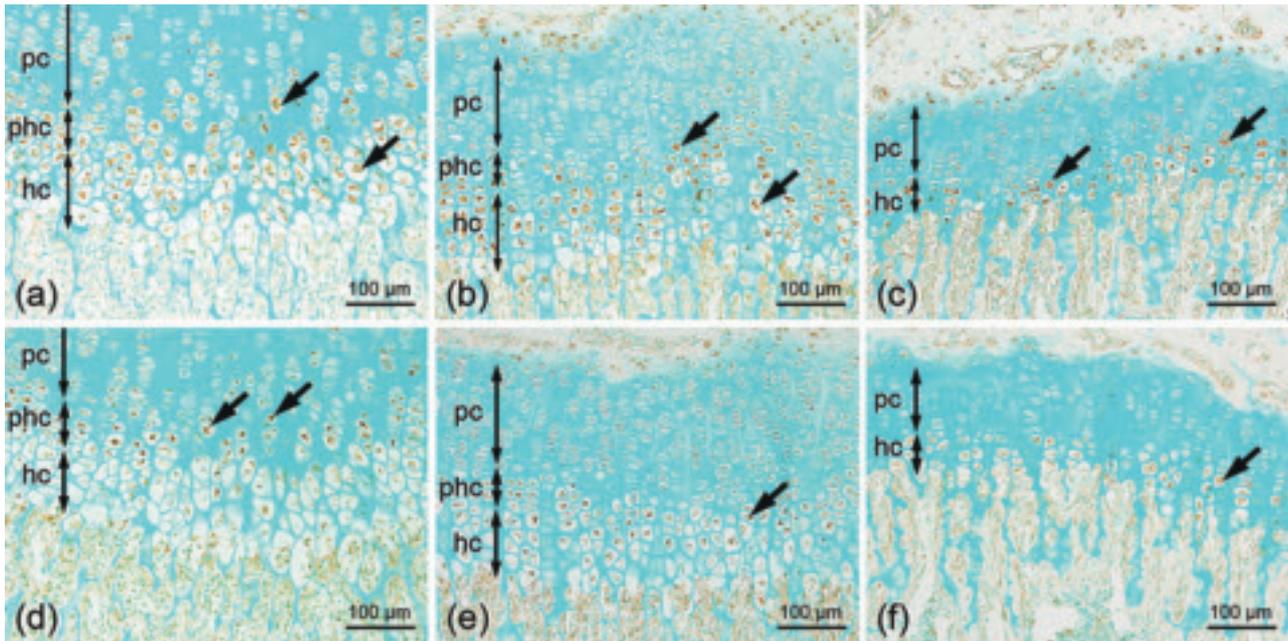
**Figure 1** Light micrographs of 1-week-old epiphyseal cartilage (a, c, g), 4-week-old growth plate (b, e, h) and 8-week-old growth plate (c, f, i). (a, b, c) Histological structure stained with hematoxylin-eosin. Prehypertrophic chondrocytes are lacking in the 8-week-old growth plate. (d, e, f) Calcification detected as brown-colored deposits after processing using von Kossa's reaction. Calcium deposits are seen only in the primary bone trabeculae of 1-week-old cartilage, but it surrounds prehypertrophic and hypertrophic chondrocytes in 4- and 8-week-old growth plates. (g, h, i) Glycogen accumulation detected using periodic acid-Schiff reaction. Reacted products are mainly seen in prehypertrophic and hypertrophic chondrocytes (arrows) of 1- and 4-week-old but not of 8-week-old cartilages. pc : proliferating chondrocytes, phc : prehypertrophic chondrocytes, hc : hypertrophic chondrocytes. Scale bar : 100  $\mu$ m.

chondrocytes (Figure 2a). In the growth plates of 4- and 8-week-old mice, the proliferating chondrocytes depressed the intensity of HIF-1 $\alpha$ , but the prehypertrophic and hypertrophic chondrocytes continued to maintain the immunoreaction (Figure 2b, c). Especially, the hypertrophic chondrocytes remained intense in 8-week-old mice (Figure 2c). On the contrary, intense immunoreactivity of HIF-2 $\alpha$  was preferentially located in the prehypertrophic chondrocytes of 1- and 4-week-old mice (Figure 2d, e). In 8-week-old mice, however, only a few hypertrophic chondrocytes displayed weak immunoreaction of HIF-2 $\alpha$  (Figure 2f).

Faint immunoreaction of GLUT1 was preferentially located in the prehypertrophic and hypertrophic chondrocytes of the cartilage of 1-, 4-, and 8-week-old mice (Figure 3a, b, c). Intense immunoreactivity of GLUT3 was found in the

prehypertrophic and hypertrophic chondrocytes of the cartilage of 1-week-old mice (Figure 3d), and further immunoreactivity of GLUT3 was intensely recognized in the prehypertrophic chondrocytes of 4-week-old and the hypertrophic chondrocytes of 8-week-old mice (Figure 3e, f). Immunoreaction of GLUT5 and GLUT9 was moderate in the prehypertrophic and hypertrophic chondrocytes of 1-week-old mice (Figure 3g, j). In contrast, the prehypertrophic chondrocytes in 4-week-old mice showed faint immunoreactivity of GLUT5 (Figure 3h), whereas they presented moderate reactivity of GLUT9 (Figure 3k). Both immunoreactivities of GLUT5 and GLUT9 were faintly observed in the hypertrophic chondrocytes of 8-week-old mice (Figure 3i, l).

Intense immunoreactivity of MCT1 was mainly located in the prehypertrophic chondrocytes of 1-week-old epiphyseal



**Figure 2** HIF-1 $\alpha$  and HIF-2 $\alpha$  immunoreactivity in the 1-week-old epiphyseal cartilage (a, d), 4-week-old growth plate (b, e) and 8-week-old growth plate (c, f). (a, b, c) HIF-1 $\alpha$  immunoreactivity remains intense. (d, e, f) Intense HIF-2 $\alpha$  immunoreactivity is located in prehypertrophic chondrocytes, but depressed in 8-week-old growth plate. pc : proliferating chondrocytes, phc : prehypertrophic chondrocytes, hc : hypertrophic chondrocytes. Arrows indicate immunopositive products. Scale bar : 100  $\mu$ m.

cartilage (Figure 4a) and declined in 4- and 8-week-old mice (Figure 4b, c). Intense MCT2 immunoreactivity was distributed in the proliferating, prehypertrophic and hypertrophic chondrocytes in 1-week-old mice (Figure 4d), whereas moderate intensity was located in the hypertrophic chondrocytes of 4- and 8-week-old mice (Figure 4e, f). Moderate immunoreactivity of MCT4 was detected in proliferating and prehypertrophic chondrocytes (Figure 4g), but the intensity declined in the chondrocytes of 4- and 8-week-old mice (Figure 4h, i).

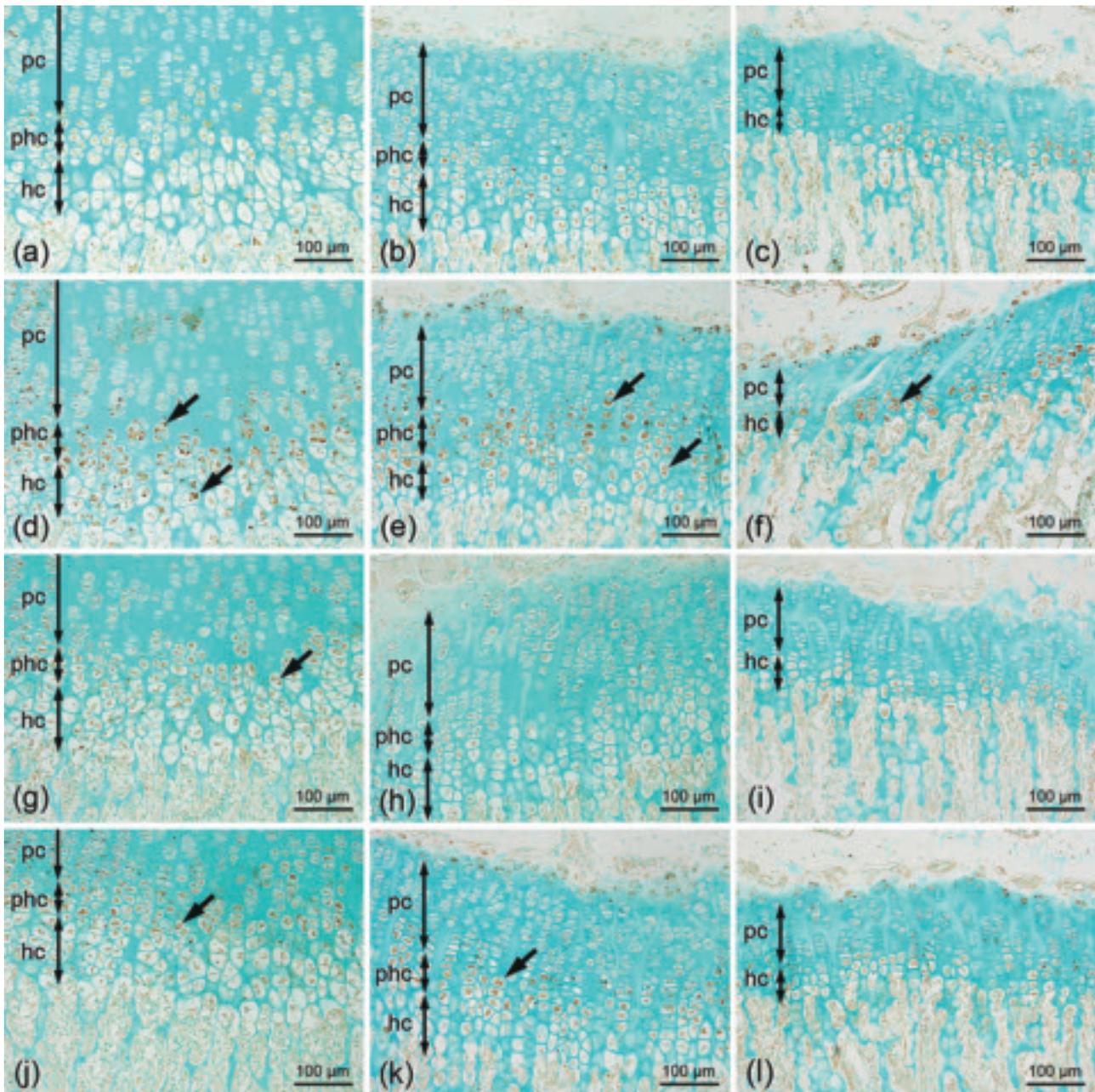
Moderate immunofluorescence of LC3B was broadly distributed in the zones from the proliferating to hypertrophic chondrocytes of 1-week-old mice (Figure 5a, d, g, j). In the growth plate of 4-week-old mice, the chondrocytes at each zone showed intense immunofluorescence of LC3B (Figure 5b, e, h, k). In 8-week-old mice, however, the hypertrophic chondrocytes continued to show intense fluorescence of LC3B, whereas the proliferating chondrocytes had remarkably decreased intensity (Figure 5c, f, i, l).

## DISCUSSION

Oxygen tension in the cartilage is relatively very low, and chondrocytes generate metabolic energy through anaerobic glycolysis (Hatori et al., 1995 ; Shapiro et al., 2005). Consequently, the chondrocytes survive, differentiate and contribute to bone growth in hypoxic conditions (Schipani, 2005 ;

Schipani et al., 2001). HIF-1 $\alpha$ , which is enhanced by hypoxia, is expressed in the early developmental stage of endochondral ossification (Pfander et al., 2003 ; Sakakura et al., 2008 ; Schipani et al., 2001). In our study, immunoreactivity of HIF-1 $\alpha$  was mainly located in prehypertrophic and hypertrophic chondrocytes at the three stages. Moderate immunoreaction was also observed in proliferating chondrocytes in the epiphyseal cartilage of 1-week-old mice. The localization of HIF-1 $\alpha$  immunoreaction was similar to those of GLUT3 and MCT2. HIF-1 $\alpha$  stabilized under hypoxic conditions contributes to a metabolic shift from the oxidative phosphorylation pathway to the oxygen-independent glycolytic pathway (Seagroves et al., 2001). Furthermore, the transcription factor is involved in regulation of GLUT expression, and expression of GLUTs is up-regulated in response to hypoxia (Semenza, 2000 ; Shannon et al., 2003 ; Pfander et al., 2003 ; Ren et al., 2008 ; Richardson et al., 2008). Thus, hypoxia during the development of epiphyseal cartilage and growth plate of mouse tibias may be closely associated with expression of GLUTs and MCTs. However, HIF-2 $\alpha$  immunoreactivity was mainly restricted in prehypertrophic chondrocytes in 1- and 4-week-old cartilage, and was weak in hypertrophic chondrocytes of 8-week-old growth plate.

In the present study, although the immunointensity ranged from faint to intense, four GLUTs were commonly localized

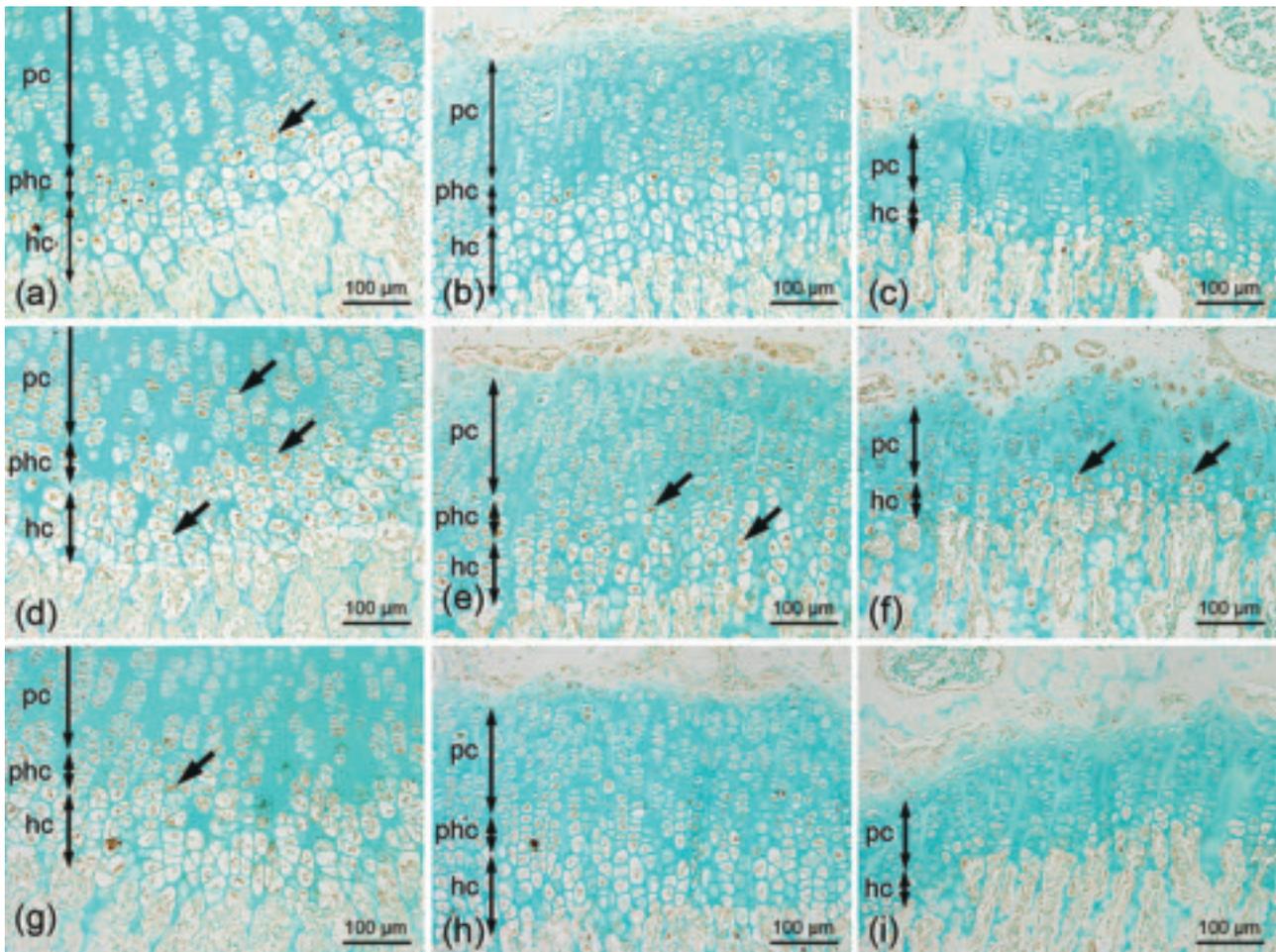


**Figure 3** Immunostaining of GLUT1, GLUT3, GLUT5 and GLUT9 in 1-week-old epiphyseal cartilage (a, d, g, j), 4-week-old growth plate (b, e, h, k) and 8-week-old growth plate (c, f, i, l). Immunoreactivity of GLUT1 (a, b, c) is faint in the prehypertrophic and hypertrophic chondrocytes of the cartilage of 1-, 4-, and 8-week-old mice. Immunoreactivity of GLUT3 (d, e, f) continues to maintain intense reactivity during chondrogenesis. Immunoreactivity of GLUT5 (g, h, i) and GLUT9 (j, k, l) decreases in 8-week-old growth plate, compared to those of 1-week-old epiphyseal cartilage and 4-week-old growth plate. pc : proliferating chondrocytes, phc : prehypertrophic chondrocytes, hc : hypertrophic chondrocytes. Arrows indicate immunopositive products. Scale bar : 100  $\mu$ m.

in prehypertrophic and hypertrophic chondrocytes of 1-week-old growth plate. The common localization was in strict correspondence with glycogen accumulation which was visualized by PAS reaction. Prehypertrophic chondrocytes may have utilized these transporters for glycogen accumulation at this stage. In contrast, GLUT3 showed intense immunoreactivity even in prehypertrophic chondrocytes of 4- and 8-week-old mice. This may indicate that chondrocytes predominantly utilize GLUT3 to survive during the cytodiffer-

entiation. Interestingly, GLUT3 is a high-affinity transporter specialized for more efficient glucose uptake at low substrate concentrations (Richardson et al., 2003 ; Mobasher et al., 2002), and the localization completely coincided with immunolocalization of HIF-1 $\alpha$ . However, glycogen accumulation shown by PAS reaction was not seen in the hypertrophic chondrocytes of 8-week-old mice, suggesting that nutrients taken into the cells may be consumed immediately.

Conversely, immunolocalization of MCT2 was similar to

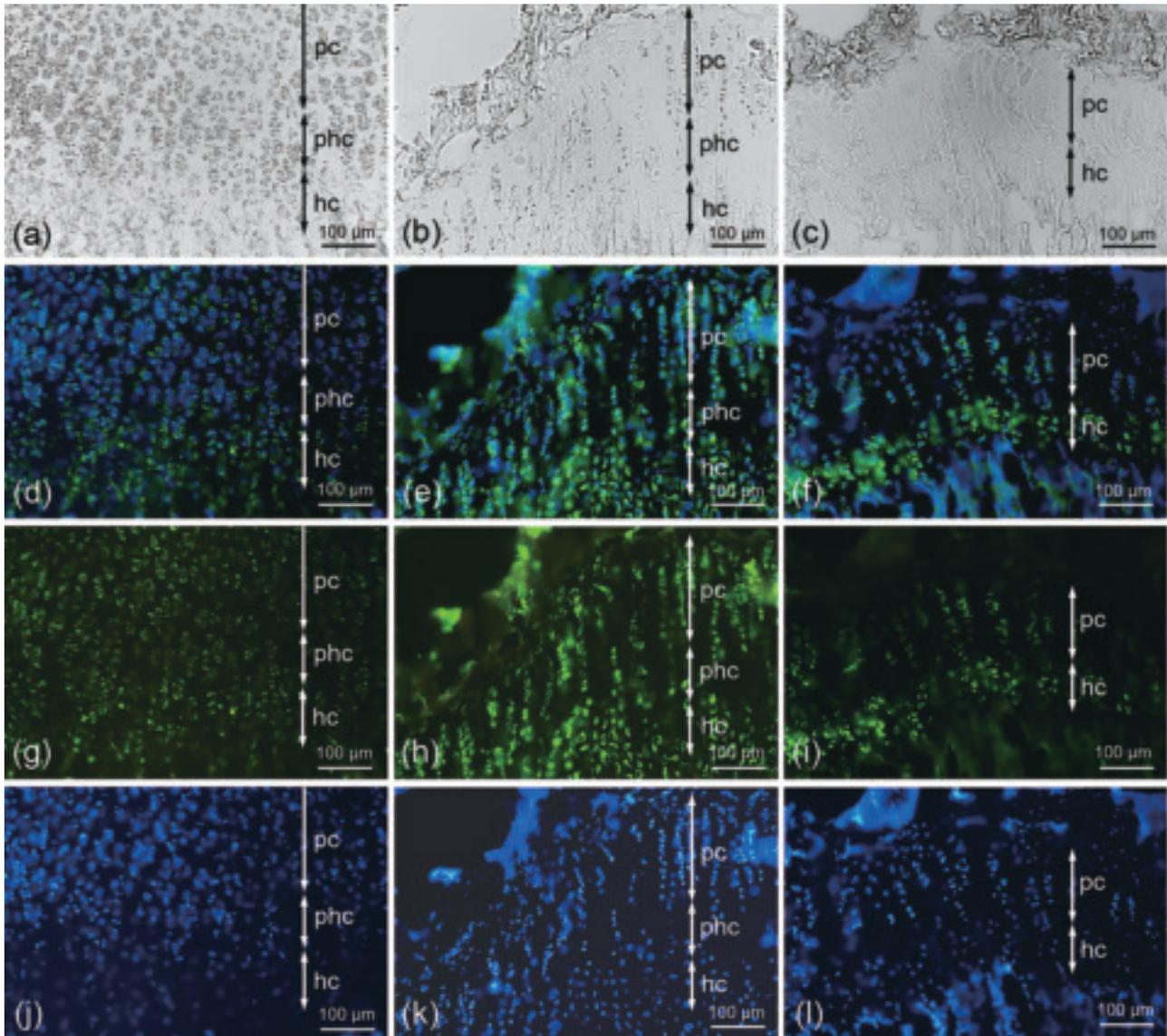


**Figure 4** Immunointensity of MCT1, MCT2 and MCT4 in 1-week-old epiphyseal cartilage (a, d, g), 4-week-old growth plate (b, e, h) and 8-week-old growth plate (c, f, i). Intense MCT1 (a, b, c) and MCT4 (g, h, i) immunostaining is seen in chondrocytes of 1-week-old epiphyseal cartilage, but declines in 4- and 8-week-old growth plates. (d, e, f) Immunoreactivity of MCT2 is moderately seen even in hypertrophic chondrocytes of 8-week-old growth plate. pc : proliferating chondrocytes, phc : prehypertrophic chondrocytes, hc : hypertrophic chondrocytes. Arrows indicate immunopositive products. Scale bar : 100  $\mu$ m.

those of HIF-1 $\alpha$  and GLUT3 in the epiphyseal cartilage of 1-week-old and growth plate of 4- and 8-week-old mice. MCT2 showed intense immunoreaction in 1-week-old mice, whereas this MCT had depressed intensity in 4- and 8-week-old mice. However, MCT2 showed moderate immunoreactivity even in hypertrophic chondrocytes of 8-week-old mice. Although the physiological role of MCT1, MCT2 and MCT4 are well known in a number of tissues and organs (Halestrap, 2012 ; Halestrap & Meredith, 2004 ; Halestrap & Wilson, 2012), the functions of these MCTs remain unknown in chondrocytes. MCT2 has a higher affinity for pyruvate and lactate than MCT1, and its expression is primarily confined to cells and tissues that take up lactic acid in significant quantities for use as respiratory fuel (Halestrap & Wilson, 2012). Accordingly, moderate MCT2 immunoreactivity in 8-week-old growth plate suggests that MCT2 may play a crucial role in survival of chondrocytes

through immediate uptake of extracellular lactate in the growth plate. In fact, our results demonstrated insufficient hypertrophy of chondrocytes in 8-week-old growth plate.

HIF-1 $\alpha$  is involved in cartilage formation with hypoxia-dependency, whereas HIF-2 $\alpha$  contributes to endochondral ossification, independent of hypoxia condition (Saito et al., 2010). The present study demonstrated that, unlike the localization of HIF-1 $\alpha$ , intense HIF-2 $\alpha$  immunoreactivity was mainly localized in prehypertrophic chondrocytes of 1- and 4-week-old cartilages. It is similar to the localization in prehypertrophic chondrocytes of growth plates in 5- or 6-week-old mouse tibias (Stewart et al., 2006). Hypoxia and HIF-1 $\alpha$  promotes chondrocyte autophagy which is a metabolic response for evading cell death (Zhang et al., 2008 ; Zhang et al., 2015). In contrast, HIF-2 $\alpha$  is a potent regulator of chondrocyte autophagy and acts as a brake to the stimulatory function of HIF-1 $\alpha$  (Bohensky et al., 2009 ; Srinivas et al.,



**Figure 5** Phase-contrast micrograph and immunofluorescence of autophagy processing marker LC3B in 1-week-old epiphyseal cartilage (a, d, g, j), 4-week-old growth plate (b, e, h, k) and 8-week-old growth plate (c, f, i, l). (a, b, c) Phase-contrast microscopic images of 1-week-old epiphyseal cartilage (a), 4-week-old growth plate (b) and 8-week-old growth plate (c). (d, e, f) Merged immunofluorescence images of LC3B (green) on nucleus of chondrocytes (blue). Unlike in 1-week-old epiphyseal cartilage (d) and 4-week-old growth plate (e), hypertrophic chondrocytes of 8-week-old growth plate (f) show intense immunofluorescence of LC3B. (g, h, i) Green colored immunofluorescence stained by LC3B antibody. (g) Moderate fluorescence is seen in all chondrocytes of 1-week-old epiphyseal cartilage. (h) Intense reaction is detected in chondrocytes of 4-week-old growth plate. (i) Immunofluorescence is decreased in proliferating chondrocytes, but hypertrophic chondrocytes show intense reactivity in 8-week-old growth plate. (j, k, l) Blue-colored nuclear staining with DAPI. pc : proliferating chondrocytes, phc : prehypertrophic chondrocytes, hc : hypertrophic chondrocytes. Scale bar : 100 μm.

2009b). Thus, the selective localization of HIF-2 $\alpha$  seems to exhibit a function different from HIF-1 $\alpha$  in chondrocyte differentiation of epiphyseal cartilage.

Suppression of HIF-2 $\alpha$  results in the induction of autophagy, whereas the staining of autophagy processing marker LC3 is seen in articular chondrocytes of 18-month-old mature and 30-month-old older mice (Bohensky et al., 2009). In 8-week-old growth plate, the hypertrophic chondrocytes displayed intense immunofluorescence of LC3B, and im-

munoreactivity of HIF-1 $\alpha$  continued to be intense, whereas that of HIF-2 $\alpha$  declined suddenly. Our results may support the activation of autophagy in a HIF-1-dependent matter (Bohensky et al., 2010 ; Srinivas et al., 2009a) and the stimulation with suppression of HIF-2 $\alpha$  (Bohensky et al., 2009).

In the present study, HIF-1 $\alpha$  immunoreactivity was recognized in prehypertrophic and hypertrophic chondrocytes of the epiphyseal cartilage and growth plate, and it was co-lo-

calized with HIF-2 $\alpha$  in prehypertrophic chondrocytes. But, immunoreactivity of HIF-2 $\alpha$  was depressed in hypertrophic chondrocytes of 8-week-old cartilage. Immunolocalization of GLUT3 completely coincided with that of HIF-1 $\alpha$  in the epiphyseal cartilage and growth plate. In addition, immunolocalization of MCTs was similar to those of HIF-1 $\alpha$  and GLUTs in the epiphyseal cartilage and growth plate. MCT2 immunoreactivity was moderate even in hypertrophic chondrocytes of 8-week-old mice. Autophagy processing marker LC3B was distributed in the zones from the proliferating to hypertrophic chondrocytes of 1- and 4-week-old cartilages, whereas it was mainly confined to hypertrophic chondrocytes of 8-week-old growth plate. Thus, these results indicated co-localization of HIF-1 $\alpha$ , GLUT3, MCT2 and LC3B in prehypertrophic and hypertrophic chondrocytes in epiphyseal cartilage and growth plates. However, HIF-2 $\alpha$  immunoreaction disappeared only in 8-week-old growth plate. We suggest that survival of chondrocytes may be controlled by HIF-1 $\alpha$  and HIF-2 $\alpha$  through uptake of nutrients and autophagy in the epiphyseal cartilage of mouse tibias.

#### Conflict of Interest

The authors declare that they have no conflict of interest.

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