Role of G-proteins in the differentiation of epiphyseal chondrocytes

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Abstract

Herein, we review the regulation of differentiation of the growth plate chondrocytes by G-proteins. In connection with this, we summarize the current knowledge regarding each family of G-protein α subunit, specifically, $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\alpha_{i/o}$. We discuss different mechanisms involved in chondrocyte differentiation downstream of G-proteins and different G-protein-coupled receptors (GPCRs) activating G-proteins in the epiphyseal chondrocytes. We conclude that among all G-proteins and GPCRs expressed by chondrocytes, $G\alpha_s$ has the most important role and prevents premature chondrocyte differentiation. Receptor for parathyroid hormone (PTHrP) appears to be the major activator of $G\alpha_s$ in chondrocytes and ablation of either one leads to accelerated chondrocyte differentiation, premature fusion of the postnatal growth plate, and ultimately short stature.

Key Words

- ▶ bone
- ▶ G proteins
- ▶ intracellular signaling
- ▶ parathyroid and bone
- ▶ skeletal

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The growth plate

The majority of bones in the skeleton are formed via the process of endochondral bone formation. During this process, a cartilage template is first formed and then replaced by true bone tissue. Formation of skeletal elements starts with condensation of mesenchymal cells with their subsequent differentiation into chondrocytes (Hall & Miyake 1995). The chondrocytes proliferate thereby increasing the size of the primary skeletal element. The cells in the center of the element stop proliferating, change their genetic program, and enlarge (hypertrophic differentiation), further facilitating growth of the skeletal element. The cartilage elements are avascular and reach a substantial size, triggering hypoxia in the middle of the skeletal element. The hypertrophic chondrocytes secrete vascular endothelial growth factor that attracts blood vessels (Zelzer *et al.* 2004). Invasion by blood vessels is accompanied by closely associated pre-osteoblasts

from the surrounding perichondrium (a thin layer of flat undifferentiated cells surrounding every cartilage element) into the center of the element (Maes *et al.* 2010). Thus, true bone tissue starts to be formed by osteoblasts in the center of cartilage anlagen, creating the primary ossification center. In the case of long bones, the secondary ossification centers are subsequently formed in a similar manner at the ends of the growing cartilage template. The secondary ossification center develops into the epiphysis and primary ossification center develops into the metaphysis and diaphysis. The cartilage remaining between the epiphysis and metaphysis forms the growth plate, a disc with spatially organized chondrocytes. The growth plate can be morphologically divided into three zones: a resting zone with round stem-like chondrocytes located toward the epiphysis, a flat cell zone with proliferating chondrocytes in the middle, and a hypertrophic zone containing differentiated enlarged chondrocytes located toward the metaphysis (Fig. 1). The

Figure 1

Structure of the growth plate. Tibial growth plate from 15-day-old mouse is shown. The section is stained with hematoxylin and eosin.

chondrocytes from the resting zone are recruited into the flat zone, where they go through several cycles of proliferation, thereby substantially increasing in number and thereafter further differentiate into hypertrophic chondrocytes. As soon as the cells cease proliferation and become pre-hypertrophic (changing from flat to round appearance), they start expressing the genes for Indian hedgehog (*IHH*) and the receptor for parathyroid hormone (*PTHr1*). *IHH* is a secreted molecule that diffuses throughout the cartilage and stimulates the production of PTH-related peptide (PTHrP) by resting cells at the top of the growth plate. The PTHrP, in turn, diffuses back to pre-hypertrophic cells, binds to the PTHr1, and inhibits chondrocyte differentiation. This inhibition increases the distance between the resting zone and the pre-hypertrophic chondrocytes and decreases the levels of *IHH*, which reach cells of the resting zone. This expansion of the growth

plate and decrease in *IHH* levels, in turn, leads to a decrease in PTHrP expression and a decrease in the distance between round and pre-hypertrophic cells. Thus, together *IHH* and PTHrP form a feedback loop, which controls the height of the growth plate (reviewed by Kronenberg (2003)). Subsequently, chondrocyte differentiation is associated with dramatic cell enlargement (hypertrophy); this cellular enlargement accounts for 59% of bone lengthening (up to 73% with associated matrix (Wilsman *et al.* 1996)). Eventually, hypertrophic chondrocytes die, allowing new bone to be formed on the cartilage template. The zone of cartilage to bone transition is called the chondro-osseous junction and the entire process is called endochondral bone formation.

Thus, the rate of bone growth depends on the following steps: recruitment of stem-like quiescent cells into flat zone, chondrocyte proliferation, differentiation from flat to hypertrophic chondrocytes, chondrocyte enlargement and, finally, replacement of hypertrophic chondrocytes by bone tissue. The rates of all these processes are tightly balanced; deregulation in the rate of any of them will lead to growth abnormalities, usually leading to short stature.

G-proteins

G-protein-coupled receptors (GPCRs), a large family of seven-transmembrane domain receptors, mediate their signaling via heterotrimeric G-protein complexes, which consists of α , β , and γ subunits. Sixteen genes encode $G\alpha$ subunits, five encode $G\beta$, and 12 genes encode $G\gamma$ subunits. When inactive, guanosine diphosphate (GDP) is bound to the α subunit, stabilizing the complex between α , β , and γ . Upon activation of the GPCR, the receptor functions as a nucleotide exchanger: GDP is replaced by the more abundant GTP. This leads to the dissociation of $G\alpha$ -GTP from $G\beta\gamma$ and allows $G\alpha$ -GTP to activate downstream effectors. The $G\beta\gamma$ dimer also may mediate signaling (reviewed in Gautam *et al.* (1998) and Oldham & Hamm (2008)), but the role of this signaling pathway in chondrocytes is largely unknown. The $G\alpha$ -GTP complex remains active until GTP is hydrolyzed. $G\alpha_s$ has an intrinsic hydrolytic activity (GTPase), which is enhanced by the regulators of G protein signaling (or RGS). There are about 30 different RGSs and their role in bone is comprehensively discussed in a recent review (Keinan *et al.* 2014).

On the basis of similarity of their α -subunits, 16 members of the $G\alpha$ subunit family are divided into four

major classes: $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\alpha_{i/o}$ (Freissmuth *et al.* 1989, Simon *et al.* 1991, Neves *et al.* 2002).

The stimulatory α subunit ($G\alpha_s$) family consists of three members: $G\alpha_s$ small and large splice isoforms of the same *GNAS1* gene and $G\alpha_{olf}$. The active $G\alpha_s$ activates adenylate cyclase, an enzyme that catalyzes the synthesis of cAMP from ATP within cells. The GPCRs are activated by many types of ligands, including glycoproteins, many peptide hormones, catecholamines, and neurotransmitters; all such types of ligands can activate $G\alpha_s$ to raise the concentration of intracellular cAMP in their target cells. cAMP works as a second messenger activating several downstream targets including protein kinase A (PKA, also known as the cAMP-dependent protein kinase or A kinase), cAMP-regulated guanine nucleotide exchange factors 1 and 2 (Epac1 and Epac2), and cyclic nucleotide-gated ion channels (Freissmuth *et al.* 1989, Simon *et al.* 1991, Kaupp & Seifert 2002, Neves *et al.* 2002).

The $G\alpha_{q/11}$ family includes four members, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15}$. Activation of $G\alpha_{q/11}$ leads to activation of PLC β , which cleaves phosphatidylinositol-4,5-bisphosphate (PIP2; also known as PTDINS(4,5)P₂) into diacylglycerol and inositol 1,4,5-trisphosphate, leading to calcium mobilization and PKC activation. In turn, PKC can activate guanine nucleotide exchange factors of the Rho family (Rho GEFs) and MAPK cascades. In addition, $G\alpha_q$ can activate AKT kinase, which modulates mTOR and NF- κ B signaling pathways (Freissmuth *et al.* 1989, Simon *et al.* 1991, Neves *et al.* 2002).

The $G\alpha_{12/13}$ family consists of two members, $G\alpha_{12}$ and $G\alpha_{13}$. Activation of these proteins induces Rho GEFs, which initiate Rho-dependent signaling through Rho-associated coiled-coil containing protein kinase (ROCK) and p38 MAPK (Freissmuth *et al.* 1989, Simon *et al.* 1991, Neves *et al.* 2002).

The $G\alpha_{i/o}$ family includes nine members: $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{0a}$, and $G\alpha_{0b}$ (splice isoforms), $G\alpha_z$, $G\alpha_{t1}$, $G\alpha_{t2}$, and $G\alpha_g$. The signaling events downstream of this family include inhibition of cAMP and calcium channels and activation of K⁺ channels, SRC, phosphoinositide 3-kinase, RAC–MEK–ERK and CDC42–PAK, and Rho-mediated signals.

The role of each of these signaling pathways in chondrocyte differentiation in the growth plate is discussed below. The data characterizing the expression of different G-proteins in the growth plate are quite limited and in this review we assume that the expression is ubiquitous, but easiest to see in pre-hypertrophic and hypertrophic chondrocytes as it is seen for $G\alpha_s$, $XL\alpha_s$, and $G\alpha_{13}$ (Chagin *et al.* 2014).

$G\alpha_s$ and chondrocyte differentiation

It appears that $G\alpha_s$ is a major participant in chondrocyte differentiation, and it inhibits the differentiation process. In humans, mutation of the $G\alpha_s$ -encoding gene, *GNAS1*, leads to Albright's hereditary osteodystrophy, which is associated with growth abnormalities, short stature, brachydactyly, and premature fusion of the growth plates (Steinbach *et al.* 1965). The mechanism underlying these abnormalities has been explored using genetic mouse models. Targeted ablation of $G\alpha_s$ in fetal (Sakamoto *et al.* 2005) or postnatal (Chagin *et al.* 2014) chondrocytes leads to dramatic acceleration of chondrocyte differentiation and cessation of longitudinal bone growth. Chimeric mice with cells deficient in the *Gnas1* gene demonstrate ectopically differentiated chondrocytes throughout the growth plate (Bastepe *et al.* 2004), further supporting the role of $G\alpha_s$ in prevention of chondrocyte differentiation. There is a splice isoform of $G\alpha_s$ (namely extra-large stimulatory alpha subunit (XL α_s)), which has an alternative exon 1, with the rest of the protein identical to $G\alpha_s$ and capable of activating the downstream cAMP signaling pathway (Klemke *et al.* 2000, Pasolli *et al.* 2000). Thus, this splice isoform may partially compensate for the lack of $G\alpha_s$. Despite being expressed by hypertrophic chondrocytes, the functional role of XL α_s seems to be negligible, because simultaneous ablation of XL α_s and $G\alpha_s$ appears phenotypically indistinguishable from $G\alpha_s$ ablation alone (Chagin *et al.* 2014).

Multiple *in vitro* experiments with administration of forskolin (adenylate cyclase activator) or 8-cAMP (long-lasting analog of cAMP) confirm the inhibitory effect of this pathway on chondrocyte differentiation (Jikko *et al.* 1996). Interestingly, activation of cAMP pathway by N⁶,2'-O-dibutyryl cAMP in differentiated cultured rabbit chondrocytes inhibits ALPase activity and collagen type X expression, markers of hypertrophic chondrocytes (Jikko *et al.* 1996), and treatment of metatarsal bones with forskolin inhibits the expression of type X collagen by hypertrophic chondrocytes (Chagin, unpublished observation). These findings suggest high plasticity of the hypertrophic differentiation stage. Altogether these observations demonstrate that chondrocyte hypertrophy is tightly controlled by the $G\alpha_s$ signaling pathway.

Canonical signaling downstream of $G\alpha_s$ includes the activation of adenylate cyclase, followed by cAMP accumulation and subsequent activation of PKA, which in turn lead to phosphorylation and activation of cAMP-response element-binding protein (CREB) transcription factor (Neves *et al.* 2002). However, in addition to PKA,

high levels of cAMP activate EPAC1 and EPAC2, whereas PKA also has multiple additional targets, including SOX9 transcription factor (Huang *et al.* 2000).

The highest levels of both $G\alpha_s$ mRNA and active PKA are observed in pre-hypertrophic and early hypertrophic chondrocytes (Chagin *et al.* 2014). Phosphorylation of CREB at serine 133, a PKA-phosphorylation site, is also observed in pre-hypertrophic chondrocytes (Long *et al.* 2001). However, the expression of dominant-negative CREB in chondrocytes delays cell differentiation (Long *et al.* 2001) in contrast to the accelerated differentiation upon genetic ablation of $G\alpha_s$. Furthermore, the levels of p-CREB were not changed upon ablation of the PTHR1 (Long *et al.* 2001), a major activator of $G\alpha_s$ signaling in chondrocytes. Thus, the $G\alpha_s$ -dependent inhibition of chondrocyte differentiation is unlikely to be mediated by CREB.

To date, there are no reports of chondrocyte-specific PKA ablation, but global inactivation of the ubiquitously expressed C- α catalytic subunit of PKA results in early postnatal lethality in 73% of animals (Skalhegg *et al.* 2002). The remaining 27% survive till weaning and showed marked growth retardation, which, according to the authors, is associated with low serum insulin-like growth factor 1 levels (Skalhegg *et al.* 2002). Unfortunately, the growth plate morphology was not presented for these animals and it is difficult to determine the exact mechanisms of growth retardation in this global PKA knockout model. In these mice, only one of the two PKA catalytic subunits was removed. Thus, the milder growth plate phenotype of these mice as compared with the chondrocyte-specific ablation of $G\alpha_s$ (lethal) is likely due to the activity of the remaining catalytic subunit C β .

An exciting downstream mechanism of $G\alpha_s$ - and PKA-dependent regulation of chondrocyte differentiation has been recently proposed by Prof. A B Lassar (Harvard Medical School). *In vitro* experiments from his group suggest that activation of PKA stimulates serine/threonine protein phosphatase 2A, which in turn removes phosphate from serine 246 of class IIa histone deacetylase HDAC4. This decreases binding of HDAC4 to 14-3-3 protein and allows translocation of HDAC4 to the nucleus, where it represses transcription activated by MEF2C (Kozhemyakina *et al.* 2009). The importance of both HDAC4 and MEF2C for chondrocytes differentiation was convincingly demonstrated *in vivo* (Vega *et al.* 2004, Arnold *et al.* 2007). This mechanism outlines a plausible downstream pathway toward $G\alpha_s$ - and PKA-mediated inhibition of chondrocyte differentiation and has now been confirmed *in vivo* (Nishimori *et al.* 2012).

Other targets downstream of $G\alpha_s$, which are likely involved in $G\alpha_s$ -regulated chondrocyte differentiation, include the suppression of the cyclin-CDK inhibitor p57; the PKA-dependent phosphorylation and activation of the transcription factor SOX9; and the suppression of the transcription of RUNX2, probably as a consequence of suppression of MEF2C action (reviewed by Kronenberg (2006)).

The large family of GPCRs is responsible for activating $G\alpha_s$. There are several GPCRs expressed by chondrocytes that can activate $G\alpha_s$. These include PTHR1 (Lee *et al.* 1993), receptors for prostaglandins (de Brum-Fernandes *et al.* 1996, Clark *et al.* 2005), membrane estrogen receptor GPER (Chagin & Savendahl 2007), RDC1 receptor (Jones *et al.* 2006), adenosine (nucleotide P2Y) receptors (Kaplan *et al.* 1996, Hoebertz *et al.* 2000), β -adrenergic receptors (Lai & Mitchell 2008), histamine H2 receptors (Fukuda *et al.* 1993), and probably others. Despite the fact that several of these receptors are known to signal through $G\alpha_s$, PTHR1 seems to be the predominant activator of $G\alpha_s$ in the growth plate. Indeed, ablation of PTHR1 either recapitulates the $G\alpha_s$ cKO phenotype or results in a more profound effect. Specifically, ablation of either $G\alpha_s$ or PTHR1 in chondrocytes during development (utilizing collagen 2 driven CRE) results in almost identical phenotypes (compare (Kobayashi *et al.* 2002) and (Sakamoto *et al.* 2005)). Postnatal ablation of PTHR1 in chondrocytes has a more profound phenotype than ablation of $G\alpha_s$ (Hirai *et al.* 2011, Chagin *et al.* 2014): ablation of PTHR1 leads to growth plate fusion, preceded by a decrease in chondrocyte proliferation, and ectopic apoptosis of stem-like chondrocytes in the resting zone, effects not observed upon ablation of $G\alpha_s$ (Chagin *et al.* 2014). Interestingly, chimeric mice containing PTHR1-deficient cells have ectopic hypertrophic chondrocytes throughout the growth plate similar to chimeric mice containing $G\alpha_s$ -deficient cells, but in contrast have an expanded zone of flat cells (Bastepe *et al.* 2004), thus again showing that ablation of PTHR1 confers a more profound phenotype than does ablation of $G\alpha_s$.

Ectopically differentiated chondrocytes, observed in the experiments with chimeric mice, suggest that the PTHR1- $G\alpha_s$ -PKA signaling pathway needs to be active in resting and flat chondrocytes in order to prevent their hypertrophic differentiation. Difficulties to detect PTHR1 mRNA in these cells might be explained by plausible downregulation of PTHR1 upon constitutive exposure of round and flat chondrocytes to PTHRP, the phenomenon described for other GPCR (Collins *et al.* 1989) and for PTHR1 at high PTH levels (Tian *et al.* 1994, Picton *et al.* 2000).

$G\alpha_q$ and $G\alpha_{11}$

The role of $G\alpha_{q/11}$ G-proteins in chondrocyte differentiation is less known. To date there are no experiments describing the genetic ablation or overexpression of either $G\alpha_q$ or G_{11} in chondrocytes. The best understanding of the $G\alpha_{q/11}$ role in chondrocyte differentiation comes from mice with *Pthr1* mutated in the way that it signals via $G\alpha_s$ but not via $G\alpha_{q/11}$ (DSEL point mutation, Guo *et al.* (2002)). Developmental delay in chondrocyte mineralization was observed in these mice (Guo *et al.* 2002), suggesting a function of $G\alpha_{q/11}$ (activated by PTHR1) opposed to that of $G\alpha_s$. This role of $G\alpha_{q/11}$ signaling is supported by *in vitro* experiments showing that pharmacological activation of PKC, the main downstream signaling pathway of $G\alpha_{q/11}$, promotes chondrocyte differentiation (Nurminsky *et al.* 2007, Matta & Mobasher 2014). On the other hand, synergism between PKA and PKC was also reported: i) inhibition of chondrocyte differentiation by prostaglandin PGE2 requires activation of both PKA and PKC (Li *et al.* 2004a) and ii) PTHRP-dependent inhibition of the transcription factor *RUNX2* requires activation of both PKA and PKC (Li *et al.* 2004b).

It is important to note that the effect of delayed mineralization is observed during the development of *Dsel* mice, whereas post-natal growth is rather normal (Guo *et al.* 2002, Chagin *et al.* 2014). Furthermore, despite opposing $G\alpha_s$ at mineralization, activation of $G\alpha_{q/11}$ by PTHR1 is needed for the survival of stem-like cells of the resting zone in the absence of $G\alpha_s$. Activation of PTHR1 is required for round cells to stay quiescent, which is mediated predominantly via $G\alpha_s$ signaling and its absence triggers proliferation of these cells whereas absence of both $G\alpha_s$ and $G\alpha_{q/11}$ pathways triggers their apoptosis (Fig. 2, Chagin *et al.* 2014). Very little is known about the regulation of this cell population and, accordingly, it is plausible that transition from quiescent round-to-flat chondrocytes is regulated differently as compared with flat-to-hypertrophic transition.

Despite revealing the role of $G\alpha_{q/11}$, activated by PTHR1, and providing valuable information of what can be expected from $G\alpha_{q/11}$ activation/inactivation, the above experiments do not entirely reveal the role of this G-protein family. Indeed, other potential GPCR can mediate their responses via $G\alpha_{q/11}$ signaling in chondrocytes. To date, genetic ablation of $G\alpha_{q/11}$ has not been performed and, accordingly, it cannot be excluded that activation of $G\alpha_{q/11}$ by other GPCRs impacts chondrocyte differentiation.

Figure 2

Receptor for parathyroid hormone maintains quiescence of stem-like chondrocytes via $G\alpha_s$. In the absence of $G\alpha_s$, stem-like chondrocytes are recruited into proliferation whereas without both $G\alpha_s$ and $G\alpha_{q/11}$ signaling these cells undergo apoptosis.

$G\alpha_{12}/G\alpha_{13}$

$G\alpha_{12}$ and $G\alpha_{13}$ mainly signal via their downstream target, RHOA (Worzel *et al.* 2008). Chondrocyte-specific ablation of RAC1, a member of the Rho family of small G proteins, leads to severe growth retardation associated with growth plate disorganization, hypocellularity, and decreased proliferation (Wang *et al.* 2007). In the ATDC5 chondrocytic cell line, RHOA stimulates cell proliferation and inhibits hypertrophic differentiation (Wang *et al.* 2004), which likely occurs via RHOA-dependent actin polymerization, which has been shown to activate SOX9 in chondrocytes (Kumar & Lassar 2009). Furthermore, PTHR1 activates $G\alpha_{12/13}$ family of G-proteins in the osteoblastic cell line UMR-106 (Singh *et al.* 2005).

Taken together, these observations suggest that $G\alpha_{12/13}$ might be involved in chondrocyte differentiation. We have ablated both $G\alpha_{12}$ and $G\alpha_{13}$ in chondrocytes (using a global $G\alpha_{12}$ KO combined with a Collagen 2 CRE-mediated ablation of floxed $G\alpha_{13}$ in chondrocytes), but found no changes either in post-natal bone growth or growth plate morphology (Chagin *et al.* 2014). This observation suggests that if existing, the role of $G\alpha_{12/13}$ family in growth plate chondrocytes is rather minor.

G α_i in chondrocytes

Proper genetic analysis of G α_i function in chondrocytes has never been carried out. In general, G α_i inhibits the cAMP pathway and opposes G α_s action (Neves *et al.* 2002). Thus, it can be predicted that G α_i would accelerate chondrocyte differentiation by inhibiting the cAMP pathway. In the growth plate, NPR-C receptor for C-type natriuretic peptide is expressed by hypertrophic chondrocytes (Yamashita *et al.* 2000) and can activate G α_i in some settings (Rose & Giles 2008). In articular chondrocytes, G α_i can be activated by chemokine receptors, which are expressed by chondrocytes (Borzi *et al.* 2000).

Conclusion

Among all G-proteins and GPCRs expressed by chondrocytes, G α_s has the most important role and prevents premature chondrocyte differentiation, whereas PTHrP is the major activator of G α_s in chondrocytes and ablation of either one leads to abrupt chondrocyte differentiation, premature fusion of the growth plate postnatally and ultimately short stature. The role of G $\alpha_{q/11}$ G-proteins is less defined and G $\alpha_{q/11}$ might have an important role in the prevention of apoptosis in the absence of G α_s and oppose its action to delay differentiation in the presence of G α_s . G $\alpha_{12/13}$ G-proteins seem have no major role in chondrocyte differentiation and bone elongation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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