Cell Reports
Article

Crosstalk of Humoral and Cell-Cell Contact-Mediated Signals in Postnatal Body Growth

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http://dx.doi.org/10.1016/j.celrep.2012.08.021

SUMMARY

The growth hormone (GH)–insulin-like growth factor 1 (IGF1) axis mediates postnatal body growth. The GH receptor has been regarded as the sole receptor that mediates the Janus kinase 2 (JAK2)/signal transducers and activators of the transcription 5B (STAT5B) signal toward IGF1 synthesis. Here, we report a signaling pathway that regulates postnatal body growth through EphA4, a member of the Eph family of receptor tyrosine kinases and a mediator of the cell-cell contact-mediated signaling. EphA4 forms a complex with the GH receptor, JAK2, and STAT5B and enhances *Igf1* expression predominantly via the JAK2-dependent pathway, with some direct effect on STAT5B. Mice with a defective *Epha4* gene have a gene dose-dependent short stature and low plasma IGF1 levels. *Igf1* messenger RNA (mRNA) in the liver and many other tissues was also significantly reduced in *Epha4*-knockout mice, whereas pituitary *Gh* mRNA and plasma GH levels were not. These findings suggest that the local cell-cell contact-mediated ephrin/EphA4 signal is as important as the humoral GH signal in IGF1 synthesis and body size determination.

INTRODUCTION

Growth hormone receptor (GHR) is present as a preformed dimer in hepatocytes and other cells. Binding of growth hormone (GH) triggers a conformational change in the extracellular domain that initiates downstream signaling (Birzniece et al., 2009; Cunningham et al., 1991; Rowlinson et al., 1998). In common with other cytokine receptors, GHR is devoid of enzymatic activity, and signal transduction is mediated by Janus kinase 2 (JAK2) (Pilecka et al., 2007). On binding of GH to the receptor, JAK2 is catalytically activated by transphosphorylation. Intracellular GH signaling appears to comprise three main pathways: the signal transducers and activators of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3 (PI-3) kinase pathway (Piwien-Pilipuk et al., 2002). Although these three pathways are activated by JAK2 transphosphorylation, the JAK-STAT pathway is regarded as the major effector of GHR signaling and is required for transcriptional regulation of insulin-like growth factor 1 (IGF1) in hepatic cells as well as locally in tissues (Kofoed et al., 2003). Among several STAT proteins, STAT5B is directly linked to the transcriptional regulation of IGF1 (Kofoed et al., 2003). Circulating IGF1 is produced predominantly in hepatocytes (Sjögren et al., 1999; Yakar et al., 1999) and is present as a ternary complex with IGF-binding protein 3 (IGFBP3) and acid-labile subunit (IGFALS) (Domené et al., 2004). The ternary complex slows the clearance of IGF1. Changes in the expression of IGFBP3 and IGFALS play an important role in modulating the growth-promoting actions of circulating IGF1, but not of local IGF1. IGFALS is produced by hepatocytes in a GH-dependent manner. In contrast, IGFBP3 is produced primarily by hepatic endothelial and Kupffer cells in the liver, although it is widely expressed in many tissues and the mechanism of IGFBP3 transcriptional regulation is not clearly defined (Binoux, 1997).

The GH-IGF1 axis is a major mediator of postnatal body growth. Defects in any of the molecules related to the GH-IGF1 axis result in short stature (Walenkamp and Wit, 2006; Woods, 2007). There are currently four known genetic causes of GH insensitivity (Woods, 2007). Homozygous mutation of the *GHR* gene is a cause of short stature (Laron syndrome), which was originally reported in patients with the classical clinical features of congenital GH deficiency and an elevated circulating GH level (Amselem et al., 1989; Godowski et al., 1989; Laron et al., 1966). Homozygous mutation of *STAT5B* was subsequently identified as causing phenotypes similar to severe GHR deficiency (Kofoed et al., 2003). Patients with *STAT5B* mutation have low levels of IGF1 and IGFBP3 and do not respond to exogenous GH administration. These genetic defects are associated with normal to mildly reduced birth weight. In contrast, *IGF1* gene deficiency causes substantial prenatal growth retardation with elevated levels of GH and normal levels of IGFBP3 and IGFALS (Woods et al., 1996). Patients with homozygous mutation of the *IGFALS* gene exhibit only a mild degree of growth retardation and have elevated GH secretion and very low circulating levels of IGF1

and IGFBP3 (Domené et al., 2004). Despite this knowledge, there appear to be many patients with short stature who have no clear molecular defects in these molecules.

Ephrin receptors (Ephs) belong to a superfamily of receptor tyrosine kinases classified into two subclasses, A and B, by their ligand-binding specificity (Kullander and Klein, 2002). The EphA receptors, with exception of EphA4, bind to ephrin-As, which are anchored to the cell membrane via a glycosylphosphatidylinositol linkage. The EphB receptors bind to ephrin-Bs, which have a transmembrane domain and a short cytoplasmic domain. EphA4 binds not only to all ephrin-As, but also to ephrin-B2 and ephrin-B3. Ephrin-Eph signaling mediates a number of functions, including classical repulsive axon guidance, boundary formation, cell migration, and proliferation via forward signaling from ephrin to Eph and reverse signaling from Eph to ephrin (Pasquale, 2008). Some signals are stimulatory, whereas others are inhibitory. Recent reports suggest that Eph receptors also regulate angiogenesis in embryonic and adult tissues (Zhang and Hughes, 2006). We have previously reported their interaction with fibroblast growth factor receptors (FGFRs) and enhancement of the canonical fibroblast growth factor signaling pathway (Sawada et al., 2010; Yokote et al., 2005). However, a complete view of the biologic functions of Ephs and ephrins has yet to be clarified.

Here, we report functions of EphA4 in JAK2-dependent and -independent STAT5B activation, leading to enhanced synthesis of IGF1. Mice defective in the *Epha4* gene showed moderate to severe short stature and low expression of *Igf1* in the liver and many other tissues. Our findings suggest that the ephrin/EphA4 and GH/GH receptor signal systems function synergistically in the production of IGF1 in multiple tissues and contribute to postnatal body and organ growth.

RESULTS

Short Stature in *Epha4*-Knockout Mice

Short stature is one of the main clinical phenotypes caused by derangement of the GH-IGF1 axis. Several genetic abnormalities have been reported among members of this signal transduction pathway, including genes for GH, GHR, STAT5B, and IGF1 (Woods, 2007). We identified short stature in $Epha4^{+/-}$ and $Epha4^{-/-}$ mice (Figure 1A). Prenatal body weight was similar among the three genotypes: $Epha4^{+/+}$, $Epha4^{+/-}$, and $Epha4^{-/-}$. However, $Epha4^{+/-}$ and $Epha4^{-/-}$ mice showed significant growth retardation compared with wild-type (WT) mice after birth. The growth retardation of $Epha4^{+/-}$ mice was milder than that of $Epha4^{-/-}$ mice. This growth deficiency was clearly present, not only in body size, but also in the skeletal system and in organs, such as the liver (Figures 1B and 1C; Table S1). These findings suggested that EphA4 might be a determinant of body size after birth.

Generalized Shrinkage of Epiphyseal Growth Plates in *Epha4*-Knockout Mice

Longitudinal growth retardation is caused by defective development of epiphyseal growth plates. As stated earlier, EphA4 interacts with FGFRs and enhances signaling via the canonical FGFR signaling pathway (Sawada et al., 2010; Yokote et al., 2005). Therefore we examined tibial epiphyseal growth plates to determine if there was shrinkage or elongation of the prehypertrophic chondrocytes, as seen in mice with mutated *Fgfr3* (Naski et al., 1998). Hematoxylin-eosin staining and in situ hybridization of *Col2a1* (collagen 2a1) messenger RNA (mRNA) of epiphyseal sections revealed severe shrinkage of all layers of growth plates in $Epha4^{-/-}$ mice (Figure 1D). This was contrary to the finding expected from inactivation of FGFR3 signaling, which shows elongation of longitudinal bones (Ornitz, 2001).

Food Intake and Plasma Levels of GH, Thyroxine, and Corticosterone Are Not Determinants of Small Body Size in *Epha4*-Knockout Mice

Malnutrition is a major cause of growth retardation. To determine if *Epha4*-targeted mice had problems with food intake, we examined daily food intake in adult mice. Weight-adjusted food intake in $Epha4^{-/-}$ and $Epha4^{+/-}$ mice was not reduced compared with WT mice (Table S1), suggesting that the amount of food intake is not a determinant of small body size in EphA4-deficient mice. Body length was also significantly different between $Epha4^{+/+}$ and $Epha4^{-/-}$ mice in both sexes (Table S1). *Epha4* knockout did not significantly alter plasma levels of GH, thyroxine, or corticosterone that might influence body size (Figure 1E).

Secretion of GH is reported to be pulsatile, such that the plasma GH level at a given time point might be misleading with respect to the state of GH production (MacLeod et al., 1991). To examine GH expression in the pituitary, we quantified the amount of *Gh* mRNA by real-time RT-PCR. Quantities were corrected for the amount of *18S* ribosomal RNA (rRNA) amplified, as reported (Iida et al., 2004). The *Gh* mRNA/*18S* rRNA values were not significantly different among the three *Epha4* genotypes in each sex or between sexes (Figure 1F).

Plasma Levels of IGF1, IGFALS, and IGFBP3 Are Reduced in *Epha4*-Knockout Mice

Plasma levels of IGF1, IGFALS, and IGFBP3 are important parameters for the function of the GH-IGF1 axis. Levels of these proteins were significantly less in $Epha4^{-/-}$ mice compared with WT mice in both sexes (Figure 2A). The plasma IGF1 level of female $Epha4^{-/-}$ mice was 49.7% that of WT mice, and that of male $Epha4^{-/-}$ mice was 36.5% that of WT mice. The plasma IGFALS level of female $Epha4^{-/-}$ mice was also reduced to 53.2% that of the control, while that of male $Epha4^{-/-}$ mice was 57.2% that of WT mice (Figure 2B). The IGFBP3 level of female $Epha4^{-/-}$ mice was 34.1% that of WT mice, and that of male $Epha4^{-/-}$ mice was 60.3% that of WT mice (Figure 2C). The low levels of these three factors in the presence of normal plasma GH level suggested that the GH-IGF1 axis in the liver is impaired because of deletion of the *Epha4* gene. The smaller liver size of $Epha4^{-/-}$ mice (Figure 1C; Table S1) might imply functional resistance at the level of GHR or IGF1 receptor (IGF1R), since EphA4, GHR, and IGF1R are all expressed in the same cell surface compartment.

Epha4 Knockout Reduces Hepatic mRNA Expression of *Igf1* and *Igfals*, but Not of *Igfbp3*, *Ghr*, or *Igf1r*

Given that approximately 75% of plasma IGF1 is produced in the liver in response to GH, we examined the hepatic expression of