Acta Biochim Biophys Sin (2008): 841-847 | © 2008 Institute of Biochemistry and Cell Biology, SIBS, CAS | All Rights Reserved 1672-9145
http://www.abbs.info; www.blackwellpublishing.com/abbs | DOI: 10.1111/j.1745-7270.2008.00465.x

Review

Ghrelin and cell differentiation

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Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is a gastric hormone that has been found to have a wide variety of biological functions. This review summarizes our current understanding of the effects of ghrelin on cell differentiation and tissue development, with an emphasis on the lineage determination of mesenchymal stem cells.

Keywords ghrelin; adipogenesis; myogenesis; osteogenesis; neurogenesis; pancreas development

Ghrelin, a 28 amino acid gastric peptide, is identified as an endogenous ligand for the "orphan" receptor, growth hormone secretagogue receptor (GHSR), in a reverse pharmacology paradigm. As *ghre* is the proto-Indo-European root of the word growth, ghrelin is named for its original function, which was to stimulate the release of growth hormone [1]. Three molecular forms of ghrelin are found in the stomach: the 28 amino acid ghrelin having n-octanoylated serine in position 3; des-acyl ghrelin, an identical peptide in which the third amino acid serine is not acylated; and the 27 amino acid des-glutamine14 ghrelin produced by alternative splicing of the ghrelin gene. Acylation appears to be essential for ghrelin's capability to stimulate the release of growth hormone, as des-acyl ghrelin does not demonstrate any endocrine functions. Once thought to be an inactive form of ghrelin, des-acyl ghrelin has been recently reported to exercise some biological activities such as acting as a survival factor for the cardiomyocyte.

Ghrelin is synthesized mainly in neuroendocrine cells (X/A-like cells in rodents and P/D1 cells in humans) of the gastric fundus, and secreted into the circulation [2]. It is also synthesized in much smaller amounts in a variety of human tissues including several areas of the brain (hypothalamus [3], hippocampus, and cortex [4]), pituitary gland [5], small intestine [1], and pancreas [6]. Ghrelin receptor GHSR is a classic seven-transmembrane G protein-coupled receptor that is linked to multiple intracellular signaling pathways, including the intracellular calcium signaling pathway. The gene encoding the *GHSR* has two splice variants: the full-length *GHSR-1a* and its truncated molecule *GHSR-1b*, which contains only five trans-membrane domains. GHSR-1a is the receptor to which ghrelin binds and through which ghrelin exerts its effects on growth hormone release [7]; the physiological function of GHSR-1b remains to be characterized.

Ever since the discovery of its first function in the stimulation of growth hormone release [8,9], ghrelin has been reported to exercise a broad array of functions including control of food intake, energy metabolism [10], modulation of cardiovascular function [11], down-regulation of cell differentiation antigen 40 expression in endothelial cells [12], regulation of lymphocyte development and cytokine secretion [13], involvement in pathoclinical profiles of digestive system cancer [14], and control of reproduction [15]. Emerging evidence also suggests that ghrelin may play a role in the regulation of cell differentiation during development. This review will focus on our current understanding of ghrelin's effects on growth and development.

Ghrelin and Fetal/perinatal Development

The behavior of ghrelin in fetal circulation and its gene expression in placenta, fetal pancreas and neonatal pancreas imply that this hormone may play an important role in the development of fetal tissue and perinatal growth. Both acylated and des-acyl ghrelin are present in fetal rat plasma at 20 d of gestation [16]. In human beings, ghrelin immuno-reactivity is present in umbilical cord blood samples as

Received: June 25, 2008 Accepted: July 17, 2008
This work was supported by grants from the National Natural Science Foundation of China (No. 30740096), and the "985" Program at Peking University (No. 985-2-097-121)
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early as week 20 of gestation. Even though maternal ghrelin is reportedly transported from maternal blood to the fetus, total ghrelin concentrations in umbilical cord veins are higher than that in maternal blood [17,18], suggesting that ghrelin is produced in the fetus. In rats, ghrelin is present in the whole fetus as early as 12 d of pregnancy [19]. Ghrelin gene levels are low in the fetal stomach of rats by 18 d of gestation [20]. Both the number of ghrelin-immunoreactive cells and plasma ghrelin levels increase significantly in the early postnatal period and reaches adult levels by 3–5 weeks. These observations suggest that the stomach may contribute negligible amounts to circulating fetal ghrelin.

In contrast, placenta may be the major source of circulating fetal ghrelin, especially in the first trimester. Expression of ghrelin is mainly contained in cytotrophoblast cells and, to a lesser extent, in syncytiotrophoblast cells. Both ghrelin mRNA and immunoreactivity have been detected in the human placenta. Expression of ghrelin in placenta decreases significantly during the course of a pregnancy and becomes undetectable at full term.

Fetal circulating ghrelin may also derive from pancreas. High levels of ghrelin gene expression are present in a fifth islet cell-ε cell of fetal pancreas, suggesting that it may be a major source of circulating fetal ghrelin [6,21]. Ghrelin-expressing cells can be detected as early as embryonic 10.5 d in the pancreases of mouse embryos. The number of these ghrelin-producing cells increases as development proceeds. Close to birth, these ghrelin-producing cells begin to localize at the periphery of developing islets and remain visible in marginal areas of the islets in the pancreases of neonates and adults [22].

The abundance of ghrelin in the fetal endocrine pancreas suggests that ghrelin may regulate the development of pancreatic β cells. This notion is supported by two genetic studies demonstrating that expansion of ghrelin-producing cells leads to the loss of insulin-producing cells during the development of pancreatic islets [21]. Reciprocal gene expression changes of insulin and ghrelin, specifically the repression of insulin and activation of ghrelin, have been observed in Nkx2.2 mutant and Pax 4 mutant mice. Within the pancreatic islet, homeodomain protein Nkx2.2 is essential for the differentiation of all insulin-producing β cells and a subset of glucagon-producing α cells. Mice lacking Nkx2.2 have relatively normal-sized islets, but a large number of cells within the mutant islet fail to produce any of the four major islet hormones. Instead, they produce ghrelin. These observations suggest that an early block in the differentiation of insulin-producing β cells in Nkx2.2 mutant mice leads to the expansion of cells that produce ghrelin, perhaps by a cell fate switch. Pax4 mutant mice also display a similar phenotype, showing the expansion of ghrelin-producing cells at the expense of β cells. Taken together, these studies suggest that insulin and ghrelin cells share a common progenitor and that Nkx2.2 and Pax4 are required for the determination and differentiation of β cells. However, it is worth noting that these studies do not demonstrate that ghrelin directly suppresses the differentiation of β cells. Indeed, ghrelin may act to promote the regeneration of β cells in streptozocin-treated newborn rats. Early administration of ghrelin may prevent the development of diabetes in disease-prone subjects after beta cell destruction [23].

Effect of Ghrelin on the Differentiation of Mesenchymal Stem Cells

Emerging evidence has indicated that ghrelin may modulate cell proliferation and differentiation. Depending on cell type, ghrelin either stimulates or inhibits cell proliferation. Mitotic effects of ghrelin have been demonstrated in preosteoblasts [24,25], neuronal precursors [26,27], preadipocytes [28, 29], cardiomyocytes [30], and the rat GH3 pituitary cell line [31]. In cell lines derived from carcinomas of the prostate [32], thyroid [33], mammary gland [34] and lung, ghrelin acts to inhibit proliferation [35]. *In vitro* studies suggest that ghrelin induces the differentiation of several types of cells, including osteoblasts, adipocytes and neurons. Ghrelin also stimulates proliferating myoblast cells to differentiate and fuse into multinucleated myotubes. Since osteoblasts, adipocytes and myocytes are all derived from common precursor cells, the mesenchymal stem cells, it is likely that ghrelin acts to determine the linage differentiation of these cells (**Fig. 1**). This concept is

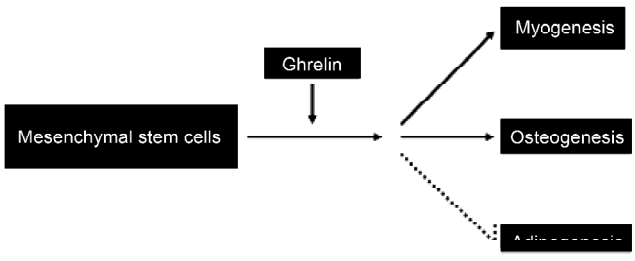

Fig. 1 Ghrelin and lineage determination of mesenchymal stem cells We propose ghrelin as an important hormone governing the cell fate switch of mesenchymal stem cells. During development, ghrelin may function to inhibit (dotted line) adipogenesis while promoting (solid lines) myogenesis and osteogenesis.

supported by studies from our laboratory and others [36–48].

Ghrelin and Adipogenesis

While the chronic administration of ghrelin in adult animals reportedly increases body weight by reducing metabolic rate and fat catabolism via a central mechanism [36], the direct effect of ghrelin on adipogenesis during development is less clear and controversial. Choi *et al* [37] have shown that exogenous ghrelin stimulates adipogenesis in primary cultures of adult rat preadipocytes, whereas Ott *et al* [38] reported that chronic treatment of SV40 large T antigen-immortalized brown adipocytes with ghrelin had no effect on adipogenesis. Using primary cultured bone marrow stromal cells, Thompson *et al* [39] reported that ghrelin and des-acyl ghrelin acts to stimulate the differentiation of adipocytes, an effect likely mediated by a yet-to-be-identified subtype of ghrelin receptor. Reasons underlying these contradictions are still unclear, but the different models used may have contributed to these conflicting observations.

To investigate the direct effect of ghrelin on adipogenesis during development, we established a stable 3T3-L1 cell line overexpressing ghrelin. Cells overexpressing ghrelin demonstrate significantly attenuated differentiation of adipocytes [28]. Expression of peroxisome proliferator-activated receptor γ (PPAR γ), which is commonly used as a marker of adipocyte differentiation, is significantly inhibited at both mRNA and protein levels. The ghrelin-mediated inhibition of adipogenesis is likely mediated by mitogen-activated protein kinase, an enzyme previously reported to regulate PPAR γ and, therefore, adipocyte differentiation [40]; both ghrelin overexpression and exogenous ghrelin stimulate the phosphorylation of mitogen-activated protein kinase. The receptor-mediated effect of ghrelin on adipogenesis appears to be an unidentified subtype. Reverse transcription-polymerase chain reaction with the primer sequence of the previously identified ghrelin receptor subtypes did not detect a signal even though ghrelin binding activity is demonstrated in both native 3T3-L1 cells and cells overexpressing ghrelin [28]. To further explore the effect of ghrelin on the development of adipose tissue, we generated a transgenic mouse that, driven by the fatty acid binding protein 4 (*FABP 4*) promoter, overexpressed ghrelin in adipose tissue. *FABP4* promoter has been reported to direct the specific expression of *Wnt 10b* in adipose tissue [41]. Our study also confirmed the specific expression of ghrelin in adipose tissue under the control of *FABP4* promoter [42]. *FABP4*-ghrelin transgenic mice demonstrate a significant decrease in the amount of adipose tissue and are resistant to obesity induced by high fat diet. These *in vivo* studies suggest that ghrelin may impair the development of adipose tissue [42].

Ghrelin and Myogenesis

Since myocytes and adipocytes are derived from a common progenitor cell, and development of muscle and adipose tissues often has a reciprocal relationship, it is likely that ghrelin has the potential to regulate the differentiation of myocytes. Studies from our laboratory and others [43, 44] provide clear evidence in support of this concept. In C2C12 cells, a mouse premyocyte cell line, overexpression of ghrelin significantly increases the differentiation of premyocytes into myocytes. Cells overexpressing ghrelin demonstrate increased myogenesis relative to control cells, as indicated by an increment in myogenic index. Expression of both Myo D, an early marker, and myosin heavy chain protein, a late marker of skeletal muscle differentiation, is elevated in cells overexpressing ghrelin compared to control cells [43]. Similar results have also been reported by Filigheddu *et al* [44]. Exogenous ghrelin and des-acyl ghrelin stimulate proliferating C2C12 skeletal myoblasts to differentiate and fuse into multinucleated myotubes by activating p38. Ghrelin's stimulatory effect on myogenesis is likely mediated by an unidentified subtype of ghrelin receptor because no signal of *GHSR-1a* mRNA has been detected in C2C12 cells, though they contain a common high-affinity binding site recognized by ghrelin.

Ghrelin and Osteogenesis

Both *in vivo* and *in vitro* studies have demonstrated that ghrelin is a potent stimulator for osteogenesis. Intraperitoneal injections of ghrelin increase bone mineral density (BMD) of the femur [45]. This effect is independent of the growth hormone because similar results have been observed in growth hormone-deficient rats. Clinical studies by Misra *et al* showed that ghrelin secretion strongly predicts BMD in healthy adolescents [46]. Our studies also showed that ghrelin promotes osteogenesis of intramembranous bone and improves the repair of calvarial bone defect in rats [47]. *In vitro*, both osteoblast cell lines and primary cultured osteoblasts respond to ghrelin with an increase in cell proliferation and differentiation [45,48]. There is still controversy about the receptor mediating the effect of ghrelin on osteogenesis. Studies by Fukushima

et al [45] demonstrated the expression of *GHSR-1a* mRNA and immunoreactivity in osteoblast cells in culture and *in situ*. In contrast, human bone does not express *GHSR-1a*. Only the *GHSR* splice variant 1b is detected in femoral bone [48]. Since the functionality and signaling pathway for GHSR-1b is unknown, the receptor mediating the ghrelin-induced osteogenesis remains to be identified.

In summary, data supporting the notion that ghrelin may be involved in the cell fate determination of mesenchymal stem cells are emerging. It is worth noting that all these data are based on the studies of precursor cells instead of the mesenchymal stem cells. Future studies should reveal the direct mechanism involved in the ghrelin-induced lineage determination of mesenchymal stem cells during development.

Ghrelin and Neurogenesis

Neurogenesis, a process through which precursor cells differentiate into a mature neuronal phenotype, persists in the circumventricular regions of the adult brain. Neurogenesis in the circumventricular region and hippocampal dentate gyrus of the adult rat nervous system has been demonstrated either under physiological conditions involving in the brain adaptation to learning, exercise and dietary restriction, or subsequent to ischemic brain injury. Global or focal cerebral ischemia has been reported to stimulate neurogenesis, typically defined by increased incorporation of bromodeoxyuridine (BrdU) into cells that express neuronal marker proteins in the subventricular zone, subgranular zone of the hippocampal dentate gyrus or cerebral cortex. Although the molecular mechanisms remain unknown, neurogenesis has been demonstrated to involve proliferation of radial cells located in the floor of cerebral ventricles [49,50].

Neurogenesis in embryos and in adult neural stem cells is regulated by a group of growth factors including stem cell factor, vascular endothelial growth factor, fibroblast growth factor, insulin-like growth factor and bone morphogenetic proteins. Our studies and others have [26, 51–59] demonstrated that ghrelin has the potential to promote neuronal development and regeneration.

Ghrelin stimulates neurogenesis in the dorsal motor nucleus of the vagus

The dorsal motor nucleus of the vagus (DMNV) contains neurons that provide the parasympathetic efferent outflow to the gastrointestinal system [60]. Both the organization and phenotype of DMNV neurons projecting into the gastrointestinal tract undergo extensive specification and reorganization in the perinatal period. The molecular mechanisms that control alteration in vagal innervations are not clear. Our studies [26] have demonstrated that ghrelin may function to control neuronal proliferation and regeneration in DMNV. Both mRNA and immunoreactivity of ghrelin receptor are detected in rat DMNV tissues. *In vivo* study demonstrates that neurogenesis exists in adult brain nuclei surrounding the fourth cerebral ventricle, including the DMNV. Although lacking active proliferation under basal conditions, the DMNV responds to perturbation induced by vagotomy with an increase in cell proliferation. Systemic administration of ghrelin significantly increases BrdU incorporation in the DMNV of adult rats with cervical vagotomy. *In vitro* exposure of cultured DMNV neurons to ghrelin significantly increases the percentage of BrdU incorporation into cells in both dose-dependent and time-dependent manners. All these data suggest that ghrelin acts directly on DMNV neurons to stimulate neurogenesis. Since the dorsal vagal complex is devoid of a brain blood barrier, the source of ghrelin responsible for stimulation of neurogenesis in the DMNV may come from the circulating blood.

Stimulation of neurogenesis in the nucleus of the solitary tract by ghrelin

The nucleus of the solitary tract (NTS) borders the fourth ventricle and possesses the characteristics of a circumventricular organ that may respond to blood-borne and cerebrospinal fluid-borne factors [51,52]. Chemical injury, such as hypoxia and hypoglycemia, results in multiple neuronal responses in the NTS, including neuronal degeneration [53] and activation of c-fos expression [54], suggesting neuronal plasticity in the NTS. Systemic administration of ghrelin significantly increases BrdU incorporation in the NTS in adult rats with cervical vagotomy [55]. Cultured NTS neurons contain immature precursor cells as evidenced by the expression of Hu protein. Exposure of cultured NTS neurons to ghrelin significantly increases the percentage of BrdU incorporation into cells in both dose-dependent and time-dependent manners. Co-localization of Hu immunoreactivity with BrdU labeling is demonstrated by double fluorescent staining, suggesting that cells labeled with BrdU are neuronal cells. Ghrelin receptor mRNA and immunoreactivity are detected in tissues from the NTS. Treating cultured NTS neurons with ghrelin receptor antagonist and calcium channel blocker abolishes the mitotic effect of ghrelin. All these studies have demonstrated that ghrelin acts directly on NTS neurons to stimulate neurogenesis via activation of GHSR-1a [55].

Ghrelin and neuronal development in spinal cord

A study by Sato *et al* [56] found that both microtubule associated protein 2 positive mature neurons and nestin positive precursor cells in fetal spinal cord expressed *GHSR* mRNA and protein. Activation of GHSR by ghrelin stimulates neuronal proliferation in cultured neurons derived from fetal spinal cord tissues. Des-acyl ghrelin also induces a significant increase in proliferation of the primary cultured neurons. Taken together, these results suggest that both ghrelin and des-acyl ghrelin are involved in neurogenesis of the fetal spinal cord [56] via both GHSR-dependent and independent mechanisms.

Neuroprotection

Neuroprotection of ghrelin has been demonstrated in ischemia/reperfusion injury [57]. Expression of GHSR-1a in rat cerebral cortex decreases significantly as a result of ischemia/reperfusion injury. Intravenous administration of ghrelin returns the *GHSR* mRNA to its normal level. Neuronal apoptosis induced by ischemia/reperfusion injury is considerably reduced by systemic administration of ghrelin. Expression of apoptosis-related molecules caspases 3 and 9 are suppressed by ghrelin, while caspase 8 remains unchanged. Neuronal damage induced by lipopolysaccharide (10 nM), glutamate (100 μM), N-methyl-D-aspartate (100 μM) or hydrogen peroxide (500 μM) is also attenuated by exogenous ghrelin [57]. All these studies suggest that ghrelin may function as a survival factor to protect cortical neurons in adult animals [57]. In the elegant study by Chung *et al*, ghrelin was also reported to protect hypothalamic neurons [58] and cortical neurons [59] from neuronal injury induced by ischemia. In addition, ghrelin acts to reduce neuronal apoptosis provoked by oxygen-glucose deprivation in cultured hypothalamic and cortical neurons. The anti-apoptotic effect of ghrelin occurs through the preservation of mitochondrial integrity.

Conclusion

The characteristics of ghrelin outlined in this review raise fascinating questions on the potential role of this hormone during cell differentiation and tissue development. Ghrelin may function as an important signal in coordinating the energy status and cell differentiation in processes such as β cell development, neurogenesis and lineage determination of mesenchymal stem cells. Future studies will soon unravel the mystery surrounding the mechanism linking the metabolic function of ghrelin to growth and development.

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