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Through their ability to regulate gene expression in most organs, glucocorticoid hormones influence numerous physiological processes and therefore are key regulators of organismal homeostasis. In bone, glucocorticoid hormones inhibit the expression of the hormone Osteocalcin for poorly understood reasons. Here we show that in a classical endocrine feedback loop, osteocalcin in return enhances the biosynthesis of glucocorticoid but also mineralocorticoid hormones (adrenal steroidogenesis) in rodents and primates. Conversely, inactivating osteocalcin signalling in adrenal glands significantly impairs adrenal growth and steroidogenesis in mice. Embryo-made osteocalcin is necessary for normal Sf1 expression in foetal adrenal cells and adrenal cell steroidogenic differentiation, it therefore determines the number of steroidogenic cells present in adrenal glands of adult animals. Embryonic not postnatal osteocalcin also governs adrenal growth, adrenal steroidogenesis, blood pressure, electrolyte equilibrium and the rise of circulating corticosterone during the acute stress response in adult offspring. This osteocalcin-dependent regulation of adrenal development and steroidogenesis occurs even in the absence of a functional hypothalamus-pituitary-adrenal axis; this explains why osteocalcin administration during pregnancy promotes adrenal growth and steroidogenesis and improves survival of adrenocorticotropic hormone signalling-deficient animals. This study reveals that a bone-derived, embryonic hormone influences lifelong adrenal functions and organismal homeostasis in the mouse.

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Embryonic osteocalcin signalling determines lifelong adrenal steroidogenesis and homeostasis in the mouse

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Running title: Osteocalcin regulation of adrenal functions
SUMMARY
Through their ability to regulate gene expression in most organs, glucocorticoid hormones influence numerous physiological processes and therefore are key regulators of organismal homeostasis. In bone, glucocorticoid hormones inhibit the expression of the hormone Osteocalcin for poorly understood reasons. Here we show that in a classical endocrine feedback loop, osteocalcin in return enhances the biosynthesis of glucocorticoid but also mineralocorticoid hormones (adrenal steroidogenesis) in rodents and primates. Conversely, inactivating osteocalcin signalling in adrenal glands significantly impairs adrenal growth and steroidogenesis in mice. Embryo-made osteocalcin is necessary for normal Sf1 expression in foetal adrenal cells and adrenal cell steroidogenic differentiation, it therefore determines the number of steroidogenic cells present in adrenal glands of adult animals. Embryonic not postnatal osteocalcin also governs adrenal growth, adrenal steroidogenesis, blood pressure, electrolyte equilibrium and the rise of circulating corticosterone during the acute stress response in adult offspring. This osteocalcin-dependent regulation of adrenal development and steroidogenesis occurs even in the absence of a functional of hypothalamus-pituitary-adrenal axis; this explains why osteocalcin administration during pregnancy promotes adrenal growth and steroidogenesis and improves survival of adrenocorticotropic hormone signalling-deficient animals. This study reveals that a bone-derived, embryonic hormone influences lifelong adrenal functions and organismal homeostasis in the mouse.

Key words: osteocalcin; adrenal glands; steroidogenesis; cell differentiation; Gpr158
INTRODUCTION

The adrenal gland, an organ of fundamental importance in mammals, is composed of two distinct areas. The inner medulla is a neural crest derivative that produces the catecholamines norepinephrine and epinephrine (1). The outer cortex is of mesodermal origin and arranged in three distinct zones, each synthesizing a different steroid hormone (1-4). The zona glomerulosa (zG) synthesizes the mineralocorticoid hormone aldosterone; the zona fasciculata (zF) synthesizes glucocorticoid hormones (GC), corticosterone in rodents, cortisol in primates; the zona reticularis (zR), which is absent in rodents synthesizes sulfate of dehydroepiandrostone (DHEAs) (1-3).

During embryogenesis, adrenal development is initiated and largely controlled by the transcription factor steroidogenic factor 1 (SF1) (1, 5). Sf1-expressing fetal adrenal cells give rise to, Gli1-positive, Sf1-negative non-steroidogenic adrenocortical progenitor cells located in the capsule surrounding the developing gland (1, 5). These adrenocortical progenitor cells differentiate into Sf1-positive aldosterone-producing cells that move centripetally. GC-producing cells originate largely from aldosterone-producing ones through lineage conversion, a process that is also dependent on Sf1 (1, 5).
Aldosterone and GC differ not only by their cells of origin but also by their regulation of synthesis and functions. Aldosterone biosynthesis is mainly regulated by extracellular potassium concentration and the renin angiotensin system in which the kidney-derived protease renin triggers multiple protein conversion events, which culminate in the generation of angiotensin 2. After binding to its receptors on cells of the zG, angiotensin 2 favors aldosterone biosynthesis (6). GC biosynthesis is controlled by hypothalamic-pituitary inputs in what defines the hypothalamic-pituitary-adrenal axis. Hypothalamus-derived corticotrophin-releasing hormone (CRH) promotes the synthesis of adrenocorticotropic hormone (ACTH) in the pituitary gland. ACTH signals in cells of the zF through the melanocortin 2 receptor (Mc2r) to favor GC synthesis and adrenal growth (7). Adrenal development occurs in part independently of pituitary influences, suggests that others yet to be identified hormones may regulate this process (8, 9).

Aldosterone regulates extracellular fluid and electrolyte homeostasis and therefore influences blood pressure (6, 10). As for GC, they affect numerous physiological processes through their ability to modulate gene expression in a wide array of cell types (11, 12). In the skeleton, GC are powerful inhibitors of Osteocalcin expression in osteoblasts, a regulation that was puzzling until evidence that osteocalcin is a hormone accumulated (13, 14). Indeed,
given the endocrine identity of osteocalcin, the inhibition of its expression by GC suggests that through a classical feedback loop osteocalcin may be a previously unappreciated physiological regulator of GC biosynthesis. That osteocalcin regulates the biosynthesis of several other hormones adds further credence to this hypothesis (13).

Here we show that osteocalcin is necessary (in mice) and sufficient (mice and monkeys) for the biosynthesis of both GC and aldosterone (adrenal steroidogenesis). During embryogenesis, osteocalcin signals in the developing adrenal glands to promote Sf1 expression, the entire differentiation program of fetal adrenal cells into non-steroidogenic adrenocortical progenitor cells and the differentiation of these latter cells into steroidogenic ones. As a result, embryo-derived osteocalcin determines the number of steroidogenic cells, adrenal growth, steroidogenesis, and several parameters of organismal homeostasis in adult offspring. None of these events and parameters of homeostasis are influenced by postnatal osteocalcin. This osteocalcin regulation of adrenal steroidogenesis occurs independently of the HPA axis and can be harnessed to induce adrenal growth and steroidogenesis and prevent perinatal death in embryos and mice lacking ACTH signaling. Hence, an embryo-derived hormone regulates adrenal steroidogenesis and organismal homeostasis postnatally.
RESULTS

Osteocalcin is sufficient to increase adrenal steroidogenesis in rodents and primates

We used gain of function experiments as a first approach to test whether osteocalcin affects GC biosynthesis because they allow addressing this question in diverse animal species. We found that regardless of the source of osteocalcin, the time of the day, the sex or the genetic background of mice, a single injection of uncarboxylated, bioactive mouse osteocalcin (osteocalcin) (30ng/g body weight) in 2 months-old (adult) wild-type (WT) mice increased circulating corticosterone 2 to 24 hours post-injection by more than 2-fold (Figure 1A-C, S1A, S1D). Since all these recombinant proteins were prepared using the same expression vector, the relatively modest differences in potency between these various preparations of recombinant osteocalcin likely reflects differences in the way the protein was prepared in distinct laboratories. Of note, the amplitude of this osteocalcin-induced rise in circulating corticosterone is similar to the one seen 30 minutes after injecting a dose of ACTH (45ng/g body weight) 20-fold lower than what is used for an ACTH test but equimolar to the dose of osteocalcin (Figure S1B).

Unexpectedly, these osteocalcin injections also increased to the same extent, 2 to 24 hours post-injection, in mice of either sex, of two different genetic backgrounds, at two different
times of the day, circulating aldosterone, the mineralocorticoid hormone synthesized by the zG of the adrenal cortex (Figure 1D-F, S1C, S1E). Consistent with this delayed increase in circulating corticosterone and aldosterone, osteocalcin injections upregulated the expression of Cyp11b1 and Cyp11b2 that encode key enzymes in the synthesis of corticosterone and aldosterone respectively (Figure S1F) (5). Osteocalcin injections also enhanced the adrenal expression of Mc2r, the ACTH receptor, and of Agtr1a and Agtr1b, the two angiotensin 2 receptors but did not affect the one of Th, which is needed to make catecholamine in the adrenal medulla (Figure S1F).

To determine whether a chronic elevation of circulating osteocalcin would also affect adrenal steroidogenesis, we analyzed Esposb/- mice, a gain of osteocalcin function model (Figure 1G) (15). We found that circulating corticosterone and aldosterone were 35 and 25% higher respectively in adult Esposb/- than in control mice (Figure 1H-I). Among all steroidogenic genes tested, only Cyp11b1, Cyp11b2, Mc2r, Agtr1a and Agtr1b had a higher expression in Esposb/- than in control adrenal glands whereas hypothalamic Crh expression, circulating ACTH and plasma renin activity were not significantly increased in Esposb/- mice (Figure S1G-J). These observations suggest that osteocalcin signals mainly in adrenal glands to promote adrenal steroidogenesis. We also observed that Esposb/- mice that lacked one
allele of *Osteocalcin* had normal circulating osteocalcin, corticosterone and aldosterone levels indicating that osteocalcin favors adrenal steroidogenesis as a bone-derived molecule (*Figure 1G, J-K*).

To broaden the significance of these observations we asked whether exogenous osteocalcin could also affect adrenal steroidogenesis in primates. We found that osteocalcin injections increased circulating cortisol and aldosterone in monkeys as well, albeit with different kinetics. Circulating cortisol rose one hour after injection, peaked at 2 hours and returned to normal 6 hours post-injection whereas circulating aldosterone increased 3-, 6- and even 24-hours post-injection (*Figure 1L-N*). On the other hand, circulating DHEAs was not affected by these injections (*Figure S1K*). Taken together these data indicate that both acute and chronic elevation of circulating osteocalcin favor adrenal steroidogenesis in mice and non-human primates and selectively increases *Cyp11b1* and *Cyp11b2* expression in adrenal glands. Based on the higher expression of their receptors, these observations also suggest that osteocalcin may also increase ACTH and angiotensin 2 signaling in adrenal glands.

**Osteocalcin signaling on adrenal cells is necessary for adrenal steroidogenesis**

That osteocalcin can increase adrenal steroidogenesis raises an even more critical question: Is it a physiological regulator of this process? Given the number of organs involved
in regulating the production of adrenal steroid hormones addressing this question requires
to identify the receptor through which osteocalcin achieves this regulation and the organ(s)
where this receptor is expressed.

The G protein coupled receptor Gprc6a mediates most functions of osteocalcin in peripheral
organs, however, its expression is undetectable in adrenal or pituitary glands (Figure 2A)
(13). In contrast, Gpr158, the receptor that transduces the osteocalcin signal in the brain, is
expressed at least one order of magnitude higher in adrenal glands than in other peripheral
tissues (Figure 2B) (16). An in-situ hybridization analysis detected Gpr158 expression in
Cyp11b1- and Cyp11b2-expressing cells of the zF and zG but not in cells of the adrenal
medulla, pituitary gland, Crh-expressing neurons, or Renin-expressing cells of the kidney in
WT mice (Figure 2C, S2A-C). Gprc6a expression was undetectable in all these cell types
(Figure 2C, S2A, S2C).

Given this pattern of expression we analyzed mice lacking Gpr158 either in all cells (Gpr158-
/-) or in adrenocortical cells, Crh-expressing hypothalamic neurons and pituitary corticotrope
cells, during development and postnatally (Gpr158Sft-/-) (17). We found that corticosterone
and aldosterone adrenal contents were significantly lower in one month-old Gpr158-/- mice
than in WT littermates (Figure S2D-E). In Gpr158Sft-/- mice, even though the gene deletion
was not complete, there was a 40 to 50% reduction in circulating corticosterone and a 22 to 34% reduction in circulating aldosterone compared to control littermates depending on their age and sex (Figure 2D-H, S2F-G). That exogenous osteocalcin failed to increase circulating corticosterone or aldosterone in Gpr158<sub>Sfr</sub>/- mice as it did in control mice, verified that osteocalcin signals through Gpr158 to promote adrenal steroidogenesis (Figure 2I-J). Circulating adrenal steroid hormones were normal in Gpr<sup>c6a</sup>-/- mice (Figure S2H). Expression of Cyp<sub>11b1</sub>, Cyp<sub>11b2</sub>, Mc2r, Agtr<sub>1a</sub> and Agtr<sub>1b</sub> was significantly lower while Crh expression, circulating ACTH levels and plasma renin activity were either not decreased or elevated in Gpr158<sub>Sfr</sub>/- adrenal glands and mice compared to control ones (Figure 2K, S2I-K). These results are consistent with the fact that Gpr158 is expressed in adrenocortical cells but not in Crh-expressing neurons, pituitary corticotrope or Renin-expressing cells of the kidney (Figure 2C, S2A, S2C). Furthermore, deleting Gpr158 from neurons, including Crh-expressing ones, but not from adrenal cells did not affect circulating corticosterone and aldosterone (Figure 3A-F) (18). These results support the notion that osteocalcin signaling through Gpr158 in cells of the adrenal cortex is necessary for adrenal steroidogenesis in the mouse.
Embryonic osteocalcin signaling is necessary for proper adrenal steroidogenesis in adult offspring

The observations presented above inferred that adrenal steroidogenesis would be hampered in Osteocalcin-/- mice. Surprisingly however, for all adrenal parameters analyzed, adult Osteocalcin-/- mice and WT littermates were indistinguishable (Figure 4A-B). One possible explanation for this observation could be that in vivo osteocalcin is not the ligand of Gpr158 that promotes adrenal steroidogenesis. Although this hypothesis cannot be excluded a priori, the fact that osteocalcin did not increase adrenal steroidogenesis in Gpr158

Sf1-/- mice, the fact also that compound heterozygous mice lacking one allele of Osteocalcin and one allele of Gpr158 in adrenal cells exhibited a deficit in adrenal steroidogenesis while single heterozygous mice did not, argue that osteocalcin is the endogenous ligand of Gpr158 in adrenal glands (Figure 2I-J, 4C-D). Therefore, we considered as an alternative explanation for this conundrum the possibility that maternal or embryonic osteocalcin might affect adrenal development to such an extent that it would disrupt adrenal steroidogenesis postnatally.

In support of this hypothesis, we found that circulating corticosterone and aldosterone were significantly lower in 8, 24 and 52 weeks-old Osteocalcin-/- mice of either sex born from a
cross between Osteocalcin-/- parents than in WT or in Osteocalcin-/- mice born from a cross between Osteocalcin+/- parents (Figure 4E-F, S4A-B). Expression of Cyp11b1, Cyp11b2, Mc2r, Agtr1a and Agtr1b was also decreased in adrenal glands of Osteocalcin-/- mice born from Osteocalcin-/- parents compared to what was observed in adrenal glands obtained from WT mice or from Osteocalcin-/- mice born from Osteocalcin+/- mothers (Figure 4G, S4C-D). In contrast, Crh expression, circulating ACTH and plasma renin activity were higher in Osteocalcin-/- mice born from Osteocalcin-/- parents than in WT or in Osteocalcin-/- mice born from Osteocalcin+/- parents (Figure 4H-I, S4E-F). Hence, Gpr158SH-/- and Osteocalcin-/- mice born from Osteocalcin-/- parents exhibit identical phenotypic and molecular adrenal abnormalities. Of note, an injection of osteocalcin increased circulating corticosterone and aldosterone in Osteocalcin-/- mice born from Osteocalcin-/- mothers (Figure S4G).

The contribution of each parent to the adrenal insufficiency phenotype observed in Osteocalcin-/- mice born from Osteocalcin-/- parents was determined by crossing Osteocalcin+/- males with Osteocalcin-/- females or Osteocalcin-/- males with Osteocalcin+/- females and measuring circulating adrenal steroid hormones in their adult Osteocalcin-/- progeny. This experiment revealed that circulating corticosterone and
aldosterone were normal in Osteocalcin-/- mice born from Osteocalcin+/+ mothers, but significantly lower in those born from Osteocalcin-/- mothers (Figure 4J-K). Furthermore, osteocalcin was not detected in blood of Osteocalcin-/- pups born from and nursed by Osteocalcin+/+ mothers indicating that no measurable quantity of maternal osteocalcin is transferred through lactation in the mouse (Figure S4H).

The data presented above established that osteocalcin must be present in the embryo’s general circulation for adrenal steroidogenesis to occur normally postnatally but did not distinguish between a maternal or an embryonic origin of this pool of osteocalcin. If maternal osteocalcin influences postnatal adrenal steroidogenesis, Osteocalcin+/+ mice born from Osteocalcin-/- mothers should have low circulating adrenal steroid hormones. If on the other hand, embryonic osteocalcin contributes to adrenal steroidogenesis, Osteocalcin+/+ mice born from Osteocalcin-/- mothers should have normal circulating adrenal steroid hormones. We observed the latter, indicating that embryonic osteocalcin influences adrenal steroidogenesis postnatally (Figure 4J-K). To define when embryonic osteocalcin signaling is needed to assure proper adrenal steroidogenesis postnatally, we crossed male and female Osteocalcin-/- mice and injected pregnant Osteocalcin-/- females once daily with osteocalcin (300ng) from E14.5 until birth. These injections normalized circulating
corticosterone and aldosterone in adult Osteocalcin-/- progeny indicating that embryonic osteocalcin signaling between E14.5 and birth exerts a lifelong influence on adrenal steroidogenesis in offspring (Figure 4L).

Embryonic osteocalcin signaling enforces homeostasis in adult offspring

If embryonic osteocalcin signaling influences adrenal steroidogenesis postnatally to such an extent, it should also affect physiological functions that are regulated by adrenal steroid and contribute in that way to the maintenance of organismal homeostasis. To test this hypothesis, we first analyzed in control and mutant mice, blood pressure, a physiological function that is regulated by adrenal steroid hormones (6).

We found that systolic and diastolic blood pressures were both significantly lower in adult Gpr158Sf1-/- and in Osteocalcin-/- mice born from Osteocalcin-/- mothers than in control mice (Figure 5A-B). Since the mothers of Gpr158Sf1-/- mice are Gpr158fl/fl this observation established that it is embryonic osteocalcin signaling that is regulating blood pressure. In contrast, systolic and diastolic blood pressures were indistinguishable between WT mice, Osteocalcin-/- mice born from Osteocalcin+/- mothers since osteocalcin from the mothers crosses the placenta and allows normal adrenal development during embryogenesis (Figure 5C) (19). The same was true in Osteocalcin-/- mice born from Osteocalcin-/-
mothers injected once daily, with osteocalcin from E14.5 to birth even though they become deprived of osteocalcin postnatally (19) (Figure 5D). Conversely, in Esp\textsubscript{osb}/- mice that have high circulating corticosterone and aldosterone, systolic and diastolic pressures were higher than in littermate controls (Figure 5E). Since the mothers of Esp\textsubscript{osb}/- mice are Esp\textsuperscript{fl/fl}, this further confirmed that it is embryonic osteocalcin that is regulating in blood pressure in adult mice (Figure 5E).

The second homeostatic parameter we analyzed was blood potassium (K\textsuperscript{+}) concentration because it is regulated by aldosterone (6). We found that blood K\textsuperscript{+} concentration was significantly higher in Gpr158\textsubscript{Sf1}/- and Osteocalcin/- mice born from Osteocalcin/- mothers than in WT, in Osteocalcin/- mice born from Osteocalcin+/- mothers or in Osteocalcin/- mice born Osteocalcin/- mothers that had received osteocalcin injections daily from E14.5 to birth (Figure 5F-I). Conversely, blood K\textsuperscript{+} concentration was lower in Esp\textsubscript{osb}/- mice that have high circulating osteocalcin and aldosterone levels (Figure 5J).

The last homeostatic function we analysed is the elevation of circulating corticosterone that is triggered by the acute stress response. We observed that following an exposure to TMT as a stressor, circulating corticosterone levels rose to significantly lower levels in Osteocalcin/- mice born from Osteocalcin/- mothers than in Osteocalcin/- mice born from
Osteocalcin +/- mothers (20) (Figure S5A). Taken together these data indicate that through its regulation of adrenal steroidogenesis, embryonic osteocalcin signalling affects several important physiological functions and contributes to the maintenance of organismal homeostasis in adult offspring. Importantly, none of these physiological functions are affected by the absence of osteocalcin postnatally (Figures 5C, 5H) since they are normal in Osteocalcin-/- mice born from Osteocalcin+/- mothers receive maternal osteocalcin that crosses the placenta, during pregnancy (19).

Embryonic osteocalcin signalling promotes adrenal cell proliferation during development and affects lifelong adrenal growth

How could osteocalcin signalling during development affect adrenal steroidogenesis so significantly in the adult offspring? To begin addressing this fundamental question, we studied adrenal cell proliferation in embryos or new-born mice lacking osteocalcin signalling in adrenal cells. While adrenal glands of Gpr158Sfr-/- E14.5 embryos were indistinguishable from those of control embryos, they were markedly smaller in E16.5 and E18.5 Gpr158Sfr-/- embryos compared to control ones (Figure 6A). This small size of adrenal glands of Gpr158Sfr-/- embryos could not be explained by a low circulating level of corticosterone since
the mothers of $Gpr158_{Sf1-/-}$ mice are $Gpr158^{f/f}$, which have normal circulating corticosterone (Figure 2E-F).

Thus, to explain this delay in adrenal gland growth in $Gpr158_{Sf1-/-}$ embryos, we studied adrenal cells apoptosis and proliferation. While apoptosis as measured by a TUNEL assay was not different in control and mutant adrenal glands at any developmental stage analyzed, (cell proliferation assessed by Ki67 staining was decreased approximately 2-fold in adrenal glands of E18.5 $Gpr158_{Sf1-/-}$ embryos compared to those of control littermates (Figure 6B-C). This decrease in cell proliferation was specific to adrenal glands as Ki67 staining was identical in the liver of E18.5 $Gpr158_{Sf1-/-}$ and control embryos (Figure S6A). In support of the notion that adrenal cell proliferation is regulated by osteocalcin signaling during development, we found that expression of Cyclins $a1, e1$ and $e2$ was markedly decreased in $Gpr158_{Sf1-/-}$ adrenal glands compared to control ones at birth (Figure 6D). This developmental deficit in adrenal cell proliferation had postnatal consequences since adrenal gland weight remained significantly lower in $Gpr158_{Sf1-/-}$ than in control mice during adulthood (Figure 6E). Adrenal gland weight and cell proliferation was also lower in adult $Osteocalcin^{-/-}$ mice born from $Osteocalcin^{-/-}$ mothers than in control mice whereas it was normal in adult $Osteocalcin^{-/-}$ mice born from $Osteocalcin^{+/-}$ mothers or from $Osteocalcin^{-/-}$
mothers that had received daily osteocalcin injections from E14.5 until birth (Figure 6F-J, S6B). Conversely, adrenal gland weight was significantly higher in Espαββ-/- mice than in control mice (Figure 6K). Thus, osteocalcin signaling during embryonic development is necessary for adrenal cells proliferation and as a result determines adrenal gland growth and size throughout life.

Embryonic osteocalcin controls the cascade of adrenal steroidogenic cell differentiation

To elucidate how osteocalcin signaling during development could affect adrenal steroidogenesis throughout life, we also analyzed the expression of genes implicated in adrenal development in WT (Figure 7A) and mutant embryos lacking either Gpr158 expression in adrenal glands or Osteocalcin expression (Figure 7B).

Sf1 expression marks both fetal adrenal cells and steroidogenic ones in the developing adrenal glands (5, 21). While adrenal Sf1 expression was similar in E14.5 Gpr158Sfr-/- and control littermate embryos, it was markedly decreased in adrenal glands of E16.5 and 18.5 Gpr158Sfr-/- embryos and in those of E18.5 Osteocalcin-/- embryos carried by Osteocalcin-/- mothers compared to adrenal glands of control embryos; the same was true for the expression of Cyp11b2 and Cyp11b1 in zG and zF respectively (Figure 7B, S7A-B).
Accordingly, aldosterone and corticosterone intra-adrenal contents were low in E18.5 $Gpr158_{Sf1-}/-$ embryos (Figure 7C). In contrast, $Cyp11b1$ and $Cyp11b2$ expression in adrenal glands was identical in $Osteocalcin-/-$ newborn pups born from $Osteocalcin+/-$ mothers and in those born from WT mice (Figure S7C).

$Sf1$-expressing fetal adrenal cells give rise to adrenocortical progenitor cells that reside in the adrenal capsule and express $Gli1$ (1, 5). That $Gli1$ expression was dramatically reduced in adrenal glands of E16.5 and 18.5 $Gpr158_{Sf1-}/-$ embryos indicated that osteocalcin signaling in $Sf1$-positive fetal adrenal cells is also needed for the generation of $Gli1$-positive adrenocortical progenitor cells (Figure 7B). In support of this notion, inactivation of $Gpr158$ in $Gli1$-positive adrenocortical progenitor cells dramatically decreased $Cyp11b1$ and $Cyp11b2$ expression in cells of the zG and zF and the intra-adrenal content of corticosterone and aldosterone in E18.5 $Gpr158_{Axin2-}/-$ embryos (Figure 7D-E, S7D-E).

Lastly, we asked whether osteocalcin signaling contributes to the differentiation of cells of the zG into cells of the zF. Wnt signaling favors the renewal and differentiation of zG cells into zF cells, and $Axin2$ is often used as a marker of Wnt signaling in adrenal cells (4, 22, 23). That the intra-adrenal contents of aldosterone and corticosterone were decreased by 45 and 33% respectively in E18.5 $Gpr158_{Axin2-}/-$ embryos compared to control ones (Figure
indicated that osteocalcin signaling in Axin2-positive cells is necessary for the differentiation of zG cells into zF cells. Taken together these gene expression and functional analyses in various mutant embryos indicate that osteocalcin signaling is needed after E14.5 for the entire cascade of adrenal steroidogenic cell differentiation including Sf1 expression in fetal adrenal cells, the generation of Gli1-positive adrenocortical progenitor cells and their differentiation into steroidogenic cells of the zG and zF.

Osteocalcin can induce adrenal growth and steroidogenesis in the absence of ACTH signaling

In addition to enhancing adrenal cell proliferation, cell differentiation and steroidogenesis, osteocalcin signaling favors Mc2r expression in adrenal glands (Figure S1F, S1G, 2K). This explains why circulating corticosterone rose significantly less in Gpr158Sf1/-/- mice that have lower Mc2r expression than in control ones during an ACTH test (Figure 8A). This positive regulation of ACTH signaling by osteocalcin raises the question as to whether osteocalcin favors adrenal growth and steroidogenesis indirectly by promoting ACTH signaling or directly and independently of its regulation of ACTH signaling in adrenal glands. To address this question, we took advantage of the unexpected observation that Gpr158 expression is increased in adrenal glands of Mc2r/-/- newborn mice (Figure 8B). In agreement with this
observation suggesting that ACTH inhibits Gpr158 expression, ACTH signaling through Mc2r decreases Gpr158 expression in adrenal glands (Figure 8C).

Others and we have observed that despite their 50% decrease in adrenal Mc2r expression, Mc2r+/- mice exhibit a paradoxical increase in their intra-adrenal content of steroid hormones (Figure 8D) (24). We tested the hypothesis that was secondary to an increase in osteocalcin signaling. In support of this hypothesis, we found that the HPA axis inhibits Gpr158 expression since the expression of this latter gene and of Cyp11b1 and Cyp11b2 was markedly higher in adrenal glands of Mc2r+/- newborn mice than in those of littermate controls (Figure 8E-F). The fact that compound heterozygous Gpr158sn+/-; Mc2r+/- newborn mice had low intra-adrenal contents of steroid hormones whereas individual heterozygous mice did not, suggested that a reason why Mc2r+/- mice have enhanced adrenal steroidogenesis is because of an increase in osteocalcin signaling (Figure 8D).

To establish whether this is indeed the case, we crossed male and female Mc2r+/- mice and delivered osteocalcin to pregnant females once a day (300ng/day, i.p.,) from E10.5 till E18.5 or birth. We then analyzed Mc2r-/- E18.5 embryos or newborn mice. We observed that adrenal glands of E18.5 Mc2r-/- embryos carried by osteocalcin-injected Mc2r+/- mothers were 30% bigger than those of E18.5 Mc2r-/- embryos carried by vehicle-injected Mc2r+/-
mothers (Figure 8G). The number of Gli1-positive adrenocortical progenitor cells and of Cyp11b1- and Cyp11b2-positive steroidogenic cells were significantly higher in E18.5 Mc2r-/- embryos carried by osteocalcin-treated Mc2r+/+ mothers than those carried by vehicle-injected Mc2r+/+ mothers (Figure 8H). When this analysis was performed in newborn mice, we found that 70% of Mc2r-/- pups born from vehicle-injected Mc2r+/+ mothers had died within 12hrs after delivery (Table 1). In contrast, 66% of Mc2r-/- pups born from osteocalcin-injected Mc2r+/+ mothers were still alive 36hrs after birth (Table 1); these latter pups had 50 and 80% higher adrenal contents of corticosterone and aldosterone respectively than Mc2r-/- newborn mice born from vehicle-injected Mc2r+/+ mothers (Figure 8I-J). To provide molecular evidence that exogenous osteocalcin could signal in adrenal glands even in the complete absence of Mc2r expression, we took advantage of the fact that the accumulation of phospho-CREB (pCREB) in adrenal glands, the active form of this transcription factor (25) is decreased in the absence of osteocalcin signaling (data not shown). We then asked whether exogenous osteocalcin could restore, even partially, the accumulation of pCREB in adrenal glands of Mc2r-null mice. As anticipated, we found that pCREB was abundantly present in WT adrenal glands and absent in adrenal glands of Mc2r-/- newborn mice whose mothers were treated with vehicle. In contrast, pCREB accumulation was restored to near
WT levels in adrenal glands of Mc2r-null newborn mice whose mothers were injected daily with osteocalcin during their pregnancy (Figure S8A). These data show that until birth, osteocalcin can signal in adrenal glands, induce adrenal growth, and promote adrenal steroidogenesis in the absence of a functional HPA axis.

**DISCUSSION**

This study was initiated to address a lingering question of bone biology: why do glucocorticoid hormones regulate Osteocalcin expression? Our working hypothesis was that the inhibition of Osteocalcin expression by GC infers that osteocalcin regulates GC biosynthesis. Gain and loss of function experiments performed in rodents and/or primates established that this is indeed the case. Embryo-derived (embryonic) osteocalcin signaling between E14.5 and birth during mouse embryogenesis is necessary, regardless of the presence or absence of a functional HPA axis, for fetal adrenal cell maintenance, differentiation of fetal adrenal cells into adrenocortical progenitor cells and differentiation of these latter cells into steroidogenic cells. As a result, embryonic osteocalcin signaling affects in adult offspring, adrenal growth, adrenal steroidogenesis, and aspects of organismal homeostasis that postnatal osteocalcin does not affect (Figure 7). Hence, an embryonic hormone determines organismal homeostasis throughout life. Along with other works this
study also identifies bone as a major regulator of steroidogenesis in the kidneys, testes, and adrenal glands (28-30).

Besides establishing that osteocalcin is sufficient to increase GC biosynthesis in rodents and non-human primates, gain of function experiments provided several additional insights (Figure 9). First, raising circulating osteocalcin increases to a similar extent circulating corticosterone and aldosterone levels, suggesting that osteocalcin regulates the biosynthesis of both adrenal steroid hormones (1). Second, adrenal glands were bigger in mice with a chronic increase in circulating osteocalcin implying that osteocalcin might promote adrenal growth. Lastly, by showing that osteocalcin up-regulates the expression of genes encoding key enzymes in adrenal steroidogenesis, but not circulating ACTH levels or plasma renin activity, these experiments suggested that osteocalcin enhances adrenal steroidogenesis principally by signaling in adrenal glands although we cannot formally exclude the possibility that it also acts post-transcriptionally.

All these contentions were verified through loss of function experiments. Adult mice lacking Gpr158, the osteocalcin receptor expressed in cells of the adrenal cortex, experience low circulating levels of both corticosterone and aldosterone and reduced adrenal weight compared to control mice. The same was true for compound heterozygous mice lacking one
allele of Osteocalcin and one allele of Gpr158. Moreover, adrenal insufficiency phenotype of Gpr158<sub>Sf1</sub>-/- mice could not be corrected by osteocalcin injections. Several arguments indicate that osteocalcin signals preferentially, if not only, in the adrenal cortex where Gpr158 is expressed. First, Gpr158 expression cannot be detected in Crh-expressing neurons, in cells of the pituitary glands or in Renin-expressing cells of the kidney. Second, expression of both Cyp11b1 and Cyp11b2 was low in Gpr158<sub>Sf1</sub>-/- adrenal glands while neither Crh expression, circulating ACTH or plasma renin activity were decreased in Gpr158<sub>Sf1</sub>-/- mice. Third, the deletion of Gpr158 in neurons including Crh-expressing ones, does not affect adrenal steroidogenesis. There are however differences between the endocrine profile of Osteocalcin/-/- mice born from Osteocalcin/-/- mothers and of Gpr158<sub>Sf1</sub>-/-/- mice. For instance, plasma ACTH levels and plasma renin activity are higher in Osteocalcin/-/- than in Gpr158<sub>Sf1</sub>-/-/- mice compared to their respective controls (compare Figure 4H-I and S2J-K). At the present time we believe that this difference reflects the fact that the deletion of Gpr158 is incomplete in this model of cell-specific gene deletion.

The lifelong regulation of adrenal growth and steroidogenesis by osteocalcin signaling is a result of these developmental functions. Indeed, adult Osteocalcin/-/- mice born from Osteocalcin/-/- mothers have lower circulating adrenal steroid hormones and smaller adrenal
glands than WT mice whereas Osteocalcin/- mice born from Osteocalcin+/− parents that become deprived of osteocalcin only after birth, and Osteocalcin/- mice born from Osteocalcin/- mothers that had received daily injections of osteocalcin from E14.5 until birth do not. Two additional evidences indicate that it is embryonic osteocalcin that determines postnatal adrenal growth and steroidogenesis in the offspring. First, adrenal growth and steroidogenesis are increased in Esposb/- mice born from Espfl/fl mothers that have normal osteocalcin levels. Second, adrenal growth and steroidogenesis are normal in Osteocalcin+/− mice born from Osteocalcin/- mothers. The importance of the lifelong influence of embryonic osteocalcin on adrenal steroidogenesis is best illustrated by the fact that adrenal steroidogenesis, blood pressure, blood K+ concentration, the ability to increase circulating corticosterone during an acute stress response and adrenal growth are all altered in adult Osteocalcin/- mice born from Osteocalcin/- mothers. Although exogenous osteocalcin can increase adrenal steroidogenesis after birth, postnatally, endogenous osteocalcin does not affect either adrenal steroidogenesis, or homeostasis in any measurable manner. Embryonic osteocalcin signaling affects postnatal adrenal growth and steroidogenesis throughout life in large part because it determines between E14.5 and birth is necessary for Sf1 expression in fetal adrenal cells, for the generation and proliferation of
Gli1- and Axin2-positive non-steroidogenic adrenocortical progenitor cells in the subcapsular zone of the developing adrenal gland and for the generation of adrenal steroidogenic cells that express Cyp11b1 or Cyp11b2. These observations raise the question of the contribution of embryonic osteocalcin to other functions of this hormone.

The available evidence not only indicate that osteocalcin signaling is necessary for adrenal development and steroidogenic functions, but that this regulation is active and can sustain some level of adrenal steroid hormone production even when the HPA axis is disrupted. The evidence supporting this statement are that adrenal steroid hormones are elevated in Mc2r+/- mice, and that exogenous osteocalcin can rescue the phenotype of Mc2r-/- embryos and newborn mice. These findings do not exclude the possibility that osteocalcin and ACTH signaling may regulate each other. For instance, osteocalcin is necessary for proper Mc2r expression in adrenal glands through molecular pathways that may include IP3 production as it is the case in hippocampal neurons, and as shown in Figures S8, CREB-dependent signaling (16). In contrast, Mc2r expression inhibits Gpr158 expression in adrenal glands through currently unknown molecular mechanisms.

Several explanations may account for why osteocalcin signaling in adrenal glands is dispensable for adrenal growth and steroidogenesis postnatally. It is possible that ACTH
signaling hampers osteocalcin signaling in adrenal glands more strongly in adult mice than
in embryos. This explanation is consistent with the significant increase in circulating ACTH
and even more so in Mc2r expression in adult animals compared to embryos (29). The
decrease in circulating osteocalcin levels in adult mice may also explain in part why the
postnatal absence of osteocalcin does not affect adrenal growth and steroidogenesis. We
believe however that the most likely explanation, is that osteocalcin signaling is necessary
for adrenal cell differentiation during embryogenesis but not for steroidogenic gene
expression per se. In agreement with this contention, when adrenal cell differentiation
occurs normally during embryogenesis, as is the case in Osteocalcin−/− mice born form
Osteocalcin+/− mothers, Cyp11b1 and Cyp11b2 expression is normal, and steroidogenesis
proceeds normally in offspring.

This study mostly performed in the mouse raise the question of the potential relevance to
human biology. Circulating osteocalcin levels are higher in men than in women whereas
circulating cortisol levels are similar in both genders (30, 31). This discrepancy is does not
affect our conclusions since the pool of osteocalcin that is important to assure proper adrenal
steroidogenesis is the embryonic one. Actually, two lines of evidence suggest that an
osteocalcin regulation of adrenal growth and steroidogenesis may exist in humans. First, a
recent genome wide association study shows that an increase of systolic or diastolic blood pressures correlated with an increase in osteocalcin levels (32). Second and more directly, osteocalcin can enhance adrenal steroidogenesis in non-human primates. That we have been unable so far to identify individuals harboring a loss of function mutation in GPR158 through the screening of multiple databases is consistent with the notion that GPR158 seems intolerant to homozygous loss of function mutations (33). On the other hand, the fact that osteocalcin signaling in the adrenal gland regulates adrenal growth and steroidogenesis provides new molecular tools in the ongoing study aiming at elucidating the pathogenesis of the still poorly understood, pathological bilateral adrenal cell proliferation (34).

The results presented in this study also impact the regulation of steroidogenesis in mammals. Indeed, the regulation of testosterone and now corticosterone and aldosterone biosynthesis by osteocalcin, along with the modulation of the 1α hydroxylation of 25 hydroxy VitaminD$_3$ by another bone-derived hormone, FGF23, identify the skeleton as an organ being necessary for the synthesis of four steroid hormones. Surprisingly, this number is like the one of steroidogenic pathways regulated by pituitary hormones, which further highlights the biological importance of bone as an endocrine organ.
The regulation of adrenal steroidogenesis by osteocalcin also illustrates to what extent this hormone acts as a regulator of other regulatory molecules regardless of whether these are peptide or steroid hormones, cytokines, or neurotransmitters (13, 20, 35). Together the site of synthesis of osteocalcin and the large number of its regulatory functions have long begged the question of why are all these functions localized in bone? The need to coordinate the huge energetic need of bone modeling and remodeling with food intake in conditions of food scarcity, explains the coordinated regulation of bone turnover and energy metabolism (36).

Moreover, osteocalcin is necessary to allow an acute stress response to proceed, it promotes cognition, mobilizes glucose and fatty acids during exercise and enhances exercise capacity (13, 20, 37). That all these functions are necessary to sense and/or escape danger suggests that osteocalcin may belong to an endocrine network orchestrating the response to danger in bony vertebrates. The role of adrenal steroid hormones in maintaining homeostasis and the regulation of their biosynthesis by osteocalcin is consistent with such a danger sensing and fighting purpose.
METHODS

Mice

Mc2r-/- (C57Bl/6J) (24), Sf1-Cre (SV129) (17), CamK2a-Cre (C57Bl/6J) (Jax strain #005359), Gli1-CreERT (Swiss Webster; C57Bl/6J mixed) (Jax strain #007913), Axin2-CreERT (C57Bl/6N) (Jax strain #018867), Ocn-/- (SV129) (38), Gpr158flox/flox (C57Bl/6J) and Gprc6a-/- mice (SV129) (16, 27) have been described previously or obtained from Jackson laboratory. For developmental deletion of Gpr158 in Gli1-CreERT or Axin2-CreERT, female floxed mice without Cre (Gpr158flox/flox; +/-) were timed-mated with male mice carrying Cre (Gpr158flox/flox; +/-Cre) and gavage-fed with one bolus of tamoxifen (4-5mg) on embryonic day 12.5 and mothers were sacrificed on E16.5 or E18.5 to collect embryos for further processing as described. For all the experiments in Figures 1-8 and S1-8 only littermate control embryos or mice were used. Wild-type (WT) mice of indicated ages for pharmacological experiments were obtained from Jackson laboratories or from Taconic laboratory.

Non-human primates

All the methods were performed in accordance with the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals India) guidelines and
regulations under the supervision of a professional Veterinarian at the primate research facility of NII, New Delhi. All animal experiments and reporting adhere to the ARRIVE guidelines. Adult female rhesus monkeys (*Macaca mulatta*) weighing 7 to 10 kg were individually housed in standard primate cages at the large animal facility, National Institute of Immunology, New Delhi, India. Animals were maintained under standard environmental conditions (24±2.1°C, 55–60% humidity, 12h:12h dark:light photoperiod) and housed individually in stainless-steel non-human primate cages. Monkeys were injected at 1000 hours with recombinant human osteocalcin (Eli Lily, Inc., USA) (13.5 ng/g of body weight) and blood samples were obtained by femoral venipuncture in Vacutainer blood collection tubes at different time points after injection. Blood was collected at room temperature, and serum was collected after centrifugation, divided into aliquots and stored at –80°C until analysis. Samples thawed were not refrozen for further hormone analysis.

**Adrenal gland analysis**

Adrenals were cleaned of excess fat, weighed, and frozen in liquid nitrogen or fixed as described. Adrenal weights were compared based on the sum of both adrenal glands, normalized to body weight. We observed no significant difference in body weight between
genotypes and combined adrenal weights (Left + right adrenal glands) per mouse have been presented relative to their body weight in main figures 1-8 and supplemental figures 1-8.

**Hormone measurements in WT or mutant mice**

Blood samples were obtained at indicated ages between 1745 hrs and 1800 hrs from facial vein within 10 sec of handling of the mice to minimize stress-induced changes in adrenal steroids. Blood was collected in serum separating tubes (Microvet 500 Z Gel, Starstedt) allowed to clot for 30 minutes and centrifuged at 12,000 rpm for 10 min at 4°C to obtain serum. Only 2 drops of blood were collected from each mouse and serum volume varied from 19-22μl per mouse. Corticosterone and aldosterone levels were determined by ELISA assays (Abcam kits) in duplicate according to the manufacturer’s instructions. For adult mice, 2μl of serum was used for corticosterone and 5μl for aldosterone measurements. Internal controls were used in each assay from a pooled serum sample collected from adult mice to calculate inter and intra-assay variations. Inter-assay variation was <8-10% and Intra-assay variation <1-2%.

**Measurement of hormone content in adrenal glands**

**Adult mice:** Fifty μl of lysate was used for corticosterone and aldosterone measurements. Internal controls were used in each assay from a pooled lysate collected from mice of
appropriate ages to calculate inter and intra-assay variations. Inter-assay variation was <5-10% and Intra-assay variation <1-4%.

**Postnatal day 1:** Pups were euthanized on ice between 1000-1200hrs. At least 3 pregnant females per genotype were used for analysis. Right adrenal glands were collected under a dissecting microscope, snap frozen in liquid nitrogen and homogenized in 100μl of lysis buffer provided with the ELISA kits for 30 seconds using a hand-held pestle in an eppendorf, 200μl more of lysis buffer added for a total volume of 300μl followed by sonication for 30 seconds on ice. Samples were centrifuged at 4°C for 5 minutes at maximum speed and supernatants were used for ELISA assays for corticosterone or aldosterone using Abcam kits in duplicate according to the manufacturer's instructions.

**Embryonic day 18.5:** Embryos were euthanized on ice between 1000-1200hrs. At least 3 pregnant females per genotype were used for analysis. Both adrenal glands were collected under a dissecting microscope, snap frozen in liquid nitrogen and homogenized in 75μl of lysis buffer provided with the ELISA kit of corticosterone for 20 seconds using a hand-held pestle in eppendorf tube followed by sonication for 10 seconds on ice. Samples were centrifuged at 4°C for 5 minutes at maximum speed and five μl of supernatants were used for ELISA assays for corticosterone or aldosterone using Abcam kits in duplicate according
to the manufacturer’s instructions. Internal controls were used in each assay from a pooled
lysate collected from embryos were used calculate inter and intra-assay variations. Inter-
assay variation was <3-8% and Intra-assay variation <1-3%.

**Osteocalcin test in WT or mutant mice and hormone measurements**

Animals were handled at least 5 days before the experiment. Animals were weighed at
1000hrs on the day of the experiment, tagged with markers and randomized. Osteocalcin
30ng/g or vehicle 0.1% BSA in PBS injection (i.p.) was done at 1200hrs or 1800hrs. Blood
samples were collected 2 hours after injection in each animal (injections were done
sequentially between 1200hrs to 1230hrs or 1800-1830hrs). Blood samples were obtained
between 1400-1430hrs or 2000-2030hrs from facial vein within 10-15 sec of handling of the
mice to minimize stress-induced changes in adrenal steroids. Different cohorts of animals
were used for vehicle and osteocalcin injections, samples were collected 1, 2, 6, and 24hrs
post injection analysis. Blood was collected in serum separating tubes (Microvet 500 Z Gel,
Starstedt) allowed to clot for 30 minutes and centrifuged at 12,000 rpm for 10 min at 4°C to
obtain serum. Only 2 drops of blood were collected from each mouse and serum volume
varied from 19-22μl per mouse. Circulating corticosterone and aldosterone were determined
by ELISA assays (Abcam kits) in duplicate according to the manufacturer's instructions. For
adult mice 2μl of serum was used for corticosterone and 5μl for aldosterone measurements. Internal controls were used in each assay from a pooled serum sample collected from adult mice to calculate inter and intra-assay variations. Inter-assay variation was <8-10% and Intra-assay variation <1-2%.

**Blood pressure measurements**

Mice of indicated genotypes in figures were weaned at 3 weeks of age, and subjected to blood pressure measurements via a non-invasive tail-cuff plethysmography method (CODA 6 Non-invasive Mouse Blood Pressure Monitor; Kent Scientific, Torrington, CT, USA) as previously described (39). Animals were adapted to the measurement process during the three days of training (15 minutes per day). For a given session, animals were introduced into Kent plexiglass holders, kept on the Kent heating stage set to the L5 temperature setting with blood pressure measurement cuffs placed on the base of the tail. After an additional acclimation on heating stage for 15 minutes, 15 consecutive blood pressure measurements were taken over the course of 30 min on the day of the experiment. The final 5 blood pressure measurements that were considered valid (e.g., no mouse movement, sufficient volume) were averaged to calculate the systolic and diastolic blood pressure. All measurements were conducted between 0800 and 1700hrs.
Measurement of potassium in plasma collected from facial vein

Enterprise Point-of-Care (EPOC) (Element POC; Heska Corporation, Barrie, Ontario) was used to measure blood potassium concentrations in WT and mutant mice. Samples were collected from the facial vein in a green top lithium-heparin tubes, mixed to inhibit clotting immediately. Any specimen with clotted blood was excluded from analysis. All analytes were measured immediately after specimen collection. Biochemical variables were measured as follows: pH, sodium (Na+), potassium (K+), ionized calcium (iCa2+), chloride ion (Cl−) and partial pressure of carbon dioxide (PCO2). Reference values (RVs) were established and confidence intervals (CIs) were calculated around the upper and lower reference limits following guidelines from the American Society for Veterinary Clinical Pathology (ASVCP). Outliers in the data were identified using Dixon and Tukey methods.

Stress

All animals of the same batch were born within an interval of 2 weeks and were kept in mixed genotype groups of 2-5 females in the same cage, at standard laboratory conditions (12 h dark/light cycle, constant room temperature and humidity, and standard lab chow and water ad libitum). For each test, mice were transported a short distance from the holding mouse facility to the testing room in their home cages. Mouse weight was between 22g and 32g.
Unless otherwise indicated baseline blood was taken 48 hours prior to onset of stress. In 
the case of the time course experiment, each time point represents a separate batch of mice 
exposed to the indicated time period of stress. Stress was delivered by an experimentalist 
blind to the genotypes or treatment of the mice under study. For 2,3,5-Trimethyl-3-thiazoline 
(TMT) stress food and water were removed immediately prior to TMT exposure. A cotton 
swab containing 10uL of TMT (Scotts Canada Ltd. 300000368) was placed in the home 
cage for 15 minutes. Serum was collected after 15 minutes of TMT exposure, and mice were 
transferred to a fresh home cage free of TMT odor.

**Gene expression analysis**

For gene expression analysis, total RNA was isolated from the whole adrenal gland through 
Qiagen RNA isolation kit from animals of each genotype, quantified with nanodrop and 
reverse transcription was performed with 1μg RNA in 20μl volume. One μl of cDNA was 
used for qRT-PCR analysis with SYBR green method (Applied biosystems) of respective 
genes and 18s ribosomal RNA was used as an internal control. cDNA for the internal control 
was diluted 50 to 500x to reach CT value within 5-6 cycles of the gene whose expression 
was being tested. *Akr1c18* was measured separately in virgin female and male mice before 
postnatal day 26 and only in virgin female mice at older ages. qRT-PCR end products were
run on a 2% agarose gels to confirm specificity of the primers. Gene expression was reanalyzed with standard qRT-PCR in the linear range of amplification and run on a 2% agarose gel to confirm the change observed through the qRT-PCR.

qPCR primer sequences used for SYBR Green-based qPCR assays were as follows:

**Cyp11b1F:** CAGATTGTGTTTGTGACGTTG; **Cyp11b1R:** CGGTTGAAGTACCATTCTGCC

**Cyp11b2F:** TGGCTGAAGATGATACAGATC; **Cyp11b2R:** CACTGTGCCTGAAAAATGGG

**Mc2rF:** ACACCAGCAAGAAAATAACTCCG; **Mc2rR:** AGGAGGACAATCAAGTTCTCCA; **Agtr1aF:** AACAGCTTGTGGGTGATCGTC; **Agtr1aR:** CATAGCGGTATAGACAGCCCA; **Agtr1bF:** TGGCTTGCTAGTTGCCCCG; **Agtr1bR:** ACCCAGCTCCAATGGGAGT; **Agtr2F:** AACTTGGCA

**CCAATGAGTCCAG; Agtr2R:** CCAAAAAGGAGTAAAGTCAGCCAAG; **Nr5a1F:** TGCAGAATGGCGACCAG; **Nr5a1R:** TACTGGACCTGGCCTGAGAT; **Ccn1F:** TGATGCTTGTCAATGCTCGAC; **Ccn1R:** AGGTCCTCCTGTACTGCTCAT; **Ccn2F:** GCCTTCACCTGGGGAC; **Ccn2R:** TCAGA

**GCTGTTACTTGTGTTTCC; Ccn1F:** GCGTACCCTGACACCAATCTC; **Ccn1R:** CTCCTTCTGCACTTCTGCTC

**GCTGTTACTTGTGTTTCC; Ccn1F:** TGGCTTGCAAGGAGCAG; **Ccn1R:** GAGGTCGCTTGCTGTAACC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG

**Ccn2F:** GCCAAGAGCCATGTGACTATCC; **Ccn2R:** CAGAGTTAGGTTAGGTTAGG
Histological and histomorphometric analysis:

At the indicated ages, adrenal glands, brains, hypothalami, kidneys and lungs were fixed in 4% PFA in PBS for 12hrs at 4°C followed by dehydration and paraffin embedding. Histological analysis was performed as described previously (23). Immunohistochemistry for phospho-CREB was performed on 7 μm thick adrenal sections using methods described previously (16).

In situ hybridization analysis

At the indicated ages, adrenal glands, brains, hypothalami, kidneys and lungs were fixed in 4% PFA in PBS for 12hrs at 4°C followed by dehydration and paraffin embedding. For embryos, pregnant mothers were sacrificed a 1000hrs, adrenal glands with kidney were
exposed by removing the viscera, fixed for 12 hours followed by dehydration and paraffin embedding. Five-7 μm sections were used for in situ hybridization analysis using ACD RNAscope kits (RNAscope® Multiplex Fluorescent Reagent Kit v2). All probes were obtained from ACD RNAscope catalog. All images were taken with identical laser settings and pseudo-color coding in the images was done for better comparative visualization.

**ACTH test in WT and Ocn-/− offspring from Ocn+/+, Ocn+/− and Ocn-/− mothers**

WT and Ocn-/− 2 months-old mice born from Ocn+/+, Ocn+/− or Ocn-/− mothers received vehicle (0.3% BSA in PBS) or recombinant ACTH (Sigma A0298, 1 μg/g) through i.p., injection at 1000hrs. Injections were done sequentially between 1000hrs to 1025hrs. Blood samples were obtained between 1030hrs to 1100hrs from facial vein within 10 sec of handling of the mice to minimize stress-induced changes in adrenal steroids. Blood was collected in serum separating tubes (Microvet 500 Z Gel, Starstedt) allowed to clot for 30 minutes and centrifuged at 12,000rpm for 10 min at 4°C to obtain serum. Only 2 drops of blood were collected from each mouse and serum volume varied between 10 to 20μl per mouse. Circulating corticosterone and aldosterone were determined by ELISA assays (Abcam kits) in duplicate according to the manufacturer’s instructions. Two μl of serum was used for corticosterone and 5μl for aldosterone measurements with biological replicates.
Internal controls were used in each assay from a pooled serum sample collected from adult mice to calculate inter and intra-assay variations. Inter-assay variation was <7-10% and Intra-assay variation <1-2%.

**Osteocalcin injections in Mc2r +/- mothers during pregnancy**

Recombinant mouse osteocalcin (300ng/day) or vehicle was injected i.p., into pregnant mothers once per day at 1700hrs from E10.5 to E18.5 or birth. When necessary, embryos were euthanized between 1100-1300hrs unless indicated otherwise for collection of tail for genotyping and adrenal glands were snap frozen for hormone measurements or gene expression or fixed in 4%PFA prepared in DEPC-treated water for *in situ* hybridization analysis.

**Osteocalcin injections in Ocn-/- and WT mothers during pregnancy**

Recombinant mouse osteocalcin (240 ng/day) or vehicle was injected i.p., into Ocn-/- or WT pregnant mothers once per day from E10.5 or E14.5 to birth. Offspring were euthanized and analyzed for all endpoints at 2 months of age for adrenal gland weight and hormones.

**Osteocalcin ELISA assay**

Mouse circulating osteocalcin was measured by ELISA as previously described (40). Plates were coated with either anti-GLA or anti-MID antibodies in Antibody Coating Buffer
(ImmunoChemistry Technologies) for 12 hours at room temperature then washed 1X (0.1% tween, 1X PBS) and coated with blocking buffer (3% BSA, 0.1% Tween, 1X PBS) for 4 hours. Blocking buffer was decanted and samples and standards were loaded and incubated at 4°C for 12 hours. The plates were then washed 5 times and incubated with an antibody directed against the C terminus of osteocalcin conjugated to HRP and incubated for 1 hour. The plate was then washed 5 times (0.1% tween, 1X PBS) and developed in 100μl 1-Step™ Ultra TMB ELISA (Cat # 34028, Pierce) and the reaction was terminated with an equivalent volume of stop solution (1N HCl). In monkeys, circulating osteocalcin was measured using a human osteocalcin ELSA from Biolegend Inc.

**ELISA assays**

Corticosterone, Aldosterone, DHEAs, Cortisol and Epinephrine ELISA kits were obtained from the Abcam and assays were performed following manufactures instructions.

**Statistical analysis**

All values are depicted as mean ± SEM. Statistical parameters including the exact value of n, post hoc test and statistical significance are reported in every figure and figure legends. Data are estimated to be statistically significant when p<0.05 using 2-tailed unpaired t test.
or 1-way ANOVA followed by Tukey’s post hoc test. Data were analyzed using Graph Pad Prism 7. All panels in Figures *p< 0.05 versus WT or control.

Study approval

All mouse experiments were carried out in accordance with protocols approved by the Columbia University Animal Ethics Committee (Protocol# AABC4500). The monkey study was approved by the Institutional Animal Ethics Committee of the National Institute of Immunology (NII) file number 25/31/2017-CPCSEA/VKY.

AUTHOR CONTRIBUTIONS

VKY and GK conceived the study in its entirety. VKY, JB, PS and PN performed experiments. VKY, JB, PS, PN and GK analyzed and interpreted the data. VKY and GK wrote the paper.

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Figure 1
Legend to Figure 1. Osteocalcin increases circulating glucocorticoids and aldosterone in mice and monkeys.

(A) Circulating corticosterone 2hrs after injections of vehicle or recombinant osteocalcin (Ocn) from different sources (30ng/g of body weight) at 1200hrs in wild-type (WT) 2 months-old Sv129 male mice. (B-C) Circulating corticosterone in 2 months-old WT Sv129 males (B), and Sv129 females (C) at different time points post osteocalcin injection. (D) Circulating aldosterone 2hrs after vehicle or osteocalcin injections at 1200hrs in 2 months-old Sv129 male WT mice. (E-F) circulating aldosterone in Sv129 male (E) and Sv129 female (F) WT mice at different time points post osteocalcin injection. (G-K) Circulating osteocalcin (G), corticosterone (H, J), and aldosterone (I, K) in WT, Espostr/- and Espostr/-;Ocn+/- mice at 1800hrs. (L-N) Circulating osteocalcin (L), cortisol (M) and aldosterone (N) at different time points after vehicle or human osteocalcin injection at 1000hrs in rhesus monkeys. Statistical analyses were conducted using 1-way ANOVA followed by Tukey’s post hoc test (A-F, G, K, M, N) or 2-tailed unpaired t test (H-J, L). * p<0.05. ns, not significant. n=6 or more each group for mice and n=4 or more for rhesus monkeys.
Legend to Figure 2. Osteocalcin signaling through Gpr158 in adrenal gland is necessary for adrenal steroidogenesis. (A-B) Expression of Gprc6a (A) and Gpr158 (B) in different tissues of WT mice (Real-time PCR). (C) In situ hybridization analysis of Gprc6a, Gpr158, Cyp11b1 and Cyp11b2 expression in WT adrenal glands. (D-H) Gpr158 expression in adrenal glands (Real-time PCR) (D), circulating corticosterone (E, 1 month-old and G, 3 months-old), and aldosterone (F, 1 month-old and H, 3 months-old) in female and male WT and Gpr158Sfl-/- mice. (I-J) Circulating corticosterone (I) and aldosterone (J) in 3 months-old WT and Gpr158Sfl-/- mice 2hrs post vehicle or osteocalcin injection. (K) Adrenal steroidogenic gene expression in WT and Gpr158Sfl-/- mice. Statistical analyses were conducted using 2-tailed unpaired t test (D-H, K) or 1-way ANOVA followed by Tukey’s post hoc test (I-J). * p<0.05. ns, not significant. n=6 or more mice per group.
Figure 3

(A) Images of hypothalami and adrenals from ROSA/Camk2a-Cre mice.

(B) Image of a histological section from a ROSA/Camk2a-Cre mouse.

(C) Gel electrophoresis showing qPCR analysis.

(D) Bar graph showing relative Gpr158 expression in hypothalami and adrenals.

(E) Bar graph showing corticosterone levels in WT and Gpr158Camk2a-Cre mice.

(F) Bar graph showing aldosterone levels in WT and Gpr158Camk2a-Cre mice.
Legend to Figure 3. Neuronal deletion of Gpr158 through CamK2a-Cre does not affect adrenal steroidogenesis

(A) Beta-galactosidase staining in whole-mount mid-brain cross section and adrenal gland of 2 months-old Camk2a-Cre+ mice crossed with ROSA reporter mice. (B) Eosin- and beta-galactosidase-stained section of adrenal gland of 2 months-old Camk2a-Cre+ mice crossed with ROSA reporter mice. (C) Recombination analysis on genomic DNA in different tissues collected from Gpr158CamK2a-/- mice. Floxed (Fl) and deletion (Del) bands are indicated. (D-F) Gpr158 expression in hypothalamus and adrenal glands (D), circulating corticosterone (E) and aldosterone (F) in 3 months-old male WT and Gpr158CamK2a-/- mice. Statistical analyses were conducted using 2-tailed unpaired t test (D-F). * p<0.05. ns, not significant. n=5 or more in each group.
Figure 4
Legend to Figure 4. Embryonic osteocalcin promotes adrenal steroidogenesis and homeostasis in offspring. (A-B) Circulating corticosterone (A) and aldosterone (B) at 1800hrs in female and male 2 months-old Osteocalcin (Ocn)-/- mice born from Ocn+/- parents and WT littermates. (C-D) Circulating corticosterone (C) and aldosterone (D) at 1800hrs in 3 months-old WT, Ocn+/-, Gpr158Sf1+/- and Ocn+/-;Gpr158Sf1+/- mice born from Ocn+/- X Gpr158Sf1+/- parents. (E-F) Circulating corticosterone (E) and aldosterone (F) at 1800hrs in 8 weeks-old Ocn+/+ and -/- female and male mice born from Ocn+/+ or Ocn-/- isogenic parents. (G-I) Adrenal steroidogenic gene expression (G), plasma ACTH levels (H) and plasma renin activity (I) in Ocn+/+ and -/- female mice born from Ocn+/+ or Ocn-/- isogenic parents. (J-K) Circulating corticosterone and aldosterone at 1800hrs in 2 months-old Ocn+/- and -/- female and male mice born from Ocn-/-(J) or Ocn+/- (K) mothers crossed with Ocn+/- or Ocn-/- fathers respectively. (L) Circulating corticosterone and aldosterone in 2 months-old Ocn+/+ and -/- mice born from Ocn+/+ or Ocn-/- mothers that received either vehicle or osteocalcin (300ng/day) from E14.5 until birth. In each panel parents are indicated on the top and progeny on the bottom. Statistical analyses were conducted using 2-tailed unpaired t test (A-B, E-K) or 1-way ANOVA followed by Tukey’s post hoc test (C-D, L). * p<0.05. ns, not significant. n=10 or more in each group except in panel G-I (n=5 or more).
Figure 5
Legend to Figure 5. Embryonic osteocalcin promotes homeostasis in offspring.

(A-J) Systolic and diastolic blood pressure and plasma potassium concentrations in 2 months-old Ocn+/+ and -/- mice born from Ocn+/+ or Ocn-/- isogenic parents (A, F), WT and Gpr158Sf1-/- mice (B, G), Ocn+/+ and -/- mice born from Ocn+/+ parents (C, H), Ocn+/+ and -/- offspring born from Ocn+/+ or Ocn-/- mothers that received either vehicle or osteocalcin (300ng/day) from E14.5 until birth (D, I) and in WT and Esposb-/- mice (E, J). Statistical analyses were conducted using 2-tailed unpaired t test (all panels). * p<0.05. ns, not significant. n=5 or more in each group.
Legend to Figure 6. Embryonic osteocalcin signaling in adrenal glands promotes cell proliferation during development.

(A) Hematoxylin and eosin-stained sections of adrenal glands of E14.5, 16.5 and 18.5 WT and Gpr158Sfr1/- embryos. (B-C) Apoptosis (TUNEL, B) and proliferation (Ki67, C) analysis in E18.5 adrenal glands from WT and Gpr158Sfr1/- embryos. (D) Cyclin gene expression in adrenal glands of WT and Gpr158Sfr1/- newborn mice. (E-K) Adrenal gland per body weight % in mouse strains of indicated genotypes and crosses. In each panel parents are indicated on the top and progeny on the bottom. Statistical analyses were conducted using 2-tailed unpaired t test (D-I, K) or 1-way ANOVA followed by Tukey’s post hoc test (J). * p<0.05. n=10 or more in each group in panels E-K; n=5 or more in each group in panels A-C.
Figure 7
Legend to Figure 7. Embryonic osteocalcin signaling in adrenal glands establishes the steroidogenic program during development.

(A) In situ hybridization analysis of Gpr158, Sf1, Cyp11b1, Cyp11b2, Gli1 and Axin2 adrenal expression in E18.5 WT embryos. (B) In situ hybridization analysis of Sf1, Cyp11b2, Cyp11b1 and Gli1 adrenal expression in E16.5 and E18.5 WT and Gpr158Sf1/-/- and in E18.5 WT and Ocn/-/- embryos. (C) Intra-adrenal content of corticosterone and aldosterone in E18.5 WT and Gpr158Sf1/-/- embryos. (D) In situ hybridization analysis of Cyp11b2 and Cyp11b1 in E18.5 WT and Gpr158Gli1/-/- embryos. (E) Intra-adrenal content of corticosterone and aldosterone in E18.5 of WT and Gpr158Gli1/-/- (E) or Gpr158Axin2/-/- (F) embryos. Statistical analyses were conducted using 2-tailed unpaired t test (C, E, F). * p<0.05. n=5 or more in each group in embryos or mice in C, E and F; n=3 or more for in situ hybridization analysis.
Figure 8
Legend to Figure 8. Osteocalcin induces adrenal steroidogenesis and growth in the absence of ACTH signaling. (A) Circulating corticosterone levels following an acute ACTH challenge at 1000hrs in adult WT and Gpr158sf1/- mice born from Gpr158fl/fl (mother) crossed with Gpr158fl/fl;Sf1-Cre+ (father) parents. (B) Gpr158 expression in adrenal glands of WT and Mc2r/- newborn mice. (C) Gpr158 adrenal expression in WT mice 2hrs after ACTH challenge. (D) Intra-adrenal corticosterone and aldosterone contents in P1 WT, Mc2r+/-, Gpr158Sf1+/- and Mc2r+/-;Gpr158Sf1+/- mice born from Mc2r+/ X Gpr158Sf1+/- parents. (E-F) Mc2r (E), Gpr158 (E), Cyp11b1 and Cyp11b2 (F) adrenal expression in WT and Mc2r/- newborn mice. (G-H) Hematoxylin and eosin-stained sections of adrenal glands (G) and in situ hybridization analysis of Gli1, Cyp11b2 and Cyp11b1 adrenal expression (H) in E18.5 WT and Mc2r/- embryos collected from Mc2r+/ mothers that received either vehicle or osteocalcin (300ng/day) from E10.5 to 18.5. (I-J) Intra-adrenal content of corticosterone (I) and aldosterone (J) in WT and Mc2r/- newborn mice born from Mc2r+/- mothers that received vehicle or osteocalcin (300ng/day) from E10.5 to until birth. Statistical analyses were conducted using 1-way ANOVA followed by Tukey’s post hoc test (A, D, I-J) or 2-tailed unpaired t test (B, C, E, F). * p<0.05. n=6 or more embryos or offspring in each group of mice.
Endogenous osteocalcin \xrightarrow{\text{Steroidogenic Differentiation}} \text{Progenitor cells} \xrightarrow{\text{Steroidogenic cells}} \text{Gpr158} \xrightarrow{\text{Sf1+}} \text{Gli1+} \xrightarrow{\text{Cyps+}} \text{Lifelong adrenal steroidogenesis and homeostasis}

Figure 9
Legend to Figure 9. Model of the regulation of adrenal steroidogenesis and postnatal homeostasis by osteocalcin.

Embryonic osteocalcin signaling in the developing adrenal gland through Gpr158 is necessary for differentiation of foetal, progenitor and steroidogenic adrenal cells as well as for the proliferation of these cells. This impacts lifelong adrenal growth and steroidogenesis and homeostasis in the offspring. Postnatally, exogenous osteocalcin can enhance steroidogenic functions in rodents and non-human primates.
Table 1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vehicle</th>
<th>Osteocalcin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mc2r+/+</td>
<td>100% (n=29)</td>
<td>100% (n=23)</td>
</tr>
<tr>
<td>Mc2r+/-</td>
<td>100% (n=52)</td>
<td>95% (n=42)</td>
</tr>
<tr>
<td>Mc2r-/-</td>
<td>100% (n=20)</td>
<td>66% (n=24)</td>
</tr>
</tbody>
</table>

Survival %: 12hrs after birth

Table 1 Legend.

Survival analysis of WT, Mc2r+/+ and Mc2r-/- mice born from Mc2r+/- mothers that received either vehicle or osteocalcin (300ng/day, i.p., daily at 1000hrs) from E10.5 to until birth.