Chapter 7
General discussion and future directions
CONTROL OF GROWTH

Growth hormone (GH) and the insulin-like growth factors (IGF-1 and -2) are key components of the endocrine growth axis and represent the major molecules controlling body growth in vertebrates. During development, there is a switch from growth controlled by nutrition to growth regulated by GH. This maturation of the growth axis occurs after hatching in oviparous vertebrates and during the perinatal period in viviparous eutherian mammals and is characterised by an upregulation in hepatic GH-receptor (GH-R) and IGF-1 expression and down regulation of pituitary GH expression. These changes allow the developing young to control its own rate of growth via negative feedback to the brain. This thesis adds a new dimension to that understanding and shows that GH-regulated growth occurs relative to developmental stage and not to the time of birth.

THE GH/IGF-1 AXIS

Prior to this study of the wallaby and the study of the brushtail possum (Saunders et al., 2003), understanding of growth axis maturation has been restricted to eutherian mammals. In the best studied species, the sheep and the rat, the GH/IGF-1 axis matures during the perinatal period. One hypothesis for the timing of transition between these growth control mechanisms in mammals is that nutrition regulated IGF production allows the embryonic and fetal stage young to grow relative to the size and nutritional capabilities of the mother in which growth must be restrained to prevent pregnancy complications while GH-regulated growth allows the young to grow to its genetically predetermined size once it is free of the uterine environment (Gluckman and Pinal, 2003). This hypothesis fits well with the growth manipulation experiments carried out by Walton and Hammond (1938) and later by Allen et al (2002a; 2002b; 2004). When pony fetuses were allowed to gestate in a more “luxurious” uterine environment (that of a thoroughbred horse), the fetuses were significantly larger at delivery, while fetuses of thoroughbred genotype gestated in a “poor” uterine environment (that of a pony) are significantly smaller at delivery. Nevertheless, during post-natal life, breeds subject to either “luxurious” or “poor” uterine manipulation still attain the normal adult size for their breed. Therefore, the accelerated or restricted growth of these fetuses in “luxurious” or “poor” uterine environments respectively is presumably a result of either increased nutrition or increased uterine space, or a combination of these factors. This logic may also be
extended to non-mammalian vertebrates. Before hatching, available nutrition and size of the egg must restrain size, but after the chick hatches, $IGF-1$ expression increases rapidly reaching peak levels within weeks (Tanaka et al., 1996).

One obvious limitation of the growth manipulation experiments in horses is that the separate influences of increased uterine size and increased nutrition on the developing fetus cannot be separated. Marsupials are unique models in which to investigate the influence of the developmental environment because they give birth to altricial young which complete most of their development after birth. Thus, if the hypothesis is correct, the need to restrain growth during pregnancy should not be an inhibiting factor in marsupial development and so a more rapid switch to GH-regulated growth in marsupials would be predicted during the perinatal period. However, this was not the case.

During pouch life in the tammar wallaby, the GH-IGF-1 axis developed gradually, similar to the process that occurs in fetal life in the sheep. Tammar young completed maturation of their growth axis around the time of first pouch exit, a stage that is developmentally equivalent to birth in precocial eutherian mammals (Renfree, 1993; Figure 36). This suggests that the switch to GH-regulated growth during development is more strongly influenced by the specific developmental program of each species and is not a process intrinsically related to birth. This relationship has now been demonstrated in two separate studies in two different marsupials (Saunders et al., 2003; this study). Furthermore, growth during pouch life in the wallaby is highly responsive to alterations in nutrition, as demonstrated by cross-fostering young of one species to a closely related species and by effectively augmenting nutrition after cross-fostering young to mothers of the same species producing later stage milk. Thus, marsupial young respond to nutrition in a similar manner to the manipulation experiments with ponies and horse fetuses (Walton and Hammond, 1938; Allen et al., 2002a, 2002b, 2004). However, what the marsupial model tells us is that it is not the size of the uterus (or pouch) that determines early growth trajectory, but access to nutrition. These separate influences of nutrition and developmental environment can be easily teased apart using a marsupial, but not a eutherian model.
This study makes clear for the first time that nutritional dependence on the mother is a critical factor in distinguishing between GH-regulated IGF production and nutrition-regulated IGF production. Once the young approaches a developmental stage where it must switch between nutritional dependence and independence, then growth axis maturation is necessary. Thus, maturation of the growth axis may be restricted or prolonged in species that provide relatively long periods of nutritional care to their young, such as humans and whales.

Nutritional regulation of growth before the development of an independent regulatory mechanism may be related to the reproductive benefit that could be gained by the mother having control over the rate of growth. Thus, during times of relative nutritional abundance or nutritional scarcity her offspring may grow faster or slower respectively. This system would ultimately allow her to produce more or fewer young over the course of her reproductive lifespan in response to favourable or unfavourable environmental conditions. This argument may also help explain why the guinea pig has lost the physiological ability for GH to stimulate hepatic IGF-1 production. Guinea pigs are one of the most precocial mammals at birth, consuming solids within 24 hours post-partum and becoming sexually mature at 3-5 weeks of age (Künkele and Trillmich, 1997). Therefore, this species may be able to do without the complex hypothalamo-pituitary control of the rate of post-natal growth. Further studies on the guinea pig may provide the answer to these questions by investigating feedback-related effects of IGF-1 in the hypothalamus and by measuring the production of IGF-1 in response to different nutritional regimens. Additional studies in the tammar are also needed to investigate whether concentrations of GH protein in the blood match the pituitary GH expression profiles as well as investigating the ontogeny of GH pulsatility during development, which has not been defined in any mammal as yet. This would require catheterization of blood vessels to get repeated samples in wallaby pouch young which represent an ideal model for investigation of this process.

The ability to “foster-forward” pouch young and effectively speed up their developmental program provides an excellent model for further investigation into growth axis maturation. If maturation of the growth axis is really a developmental process, then young in whom development is hastened should also exhibit faster maturation of their growth axes in line with the above predictions. The sucking
regime of the pouch young also poses interesting comparative questions in relation to nutrient partitioning between marsupial and eutherian mammals during development. While the young is continuously attached to the teat during early post-natal life, it is not passively attached and may be able to regulate nutrient intake in a fashion different from eutherian mammals which is largely passive, and relies on the placenta during equivalent stages. We may yet find in marsupials the presence of imprinted genes that limit the sequestering of resources by the pouch young from the mother such as those that have been identified in eutherian and marsupial placentas.

GHRELIN

Ghrelin is a potent stimulant of GH secretion from somatotroph cells of the anterior pituitary gland and is secreted from the stomach in response to appetite. Ghrelin may also be required to stimulate appetite in marsupials which must feed from a much earlier developmental stage than most eutherian mammals. Ghrelin is therefore a link between nutrition and growth during development because it can stimulate production of GH. However, plasma GH concentrations increase during early fetal life in eutherians and remain elevated until late fetal life when the growth axis matures. In contrast, ghrelin concentrations do not increase until middle to late fetal life in the human and late fetal life in the rat, so the interaction between ghrelin and GH during development is unclear.

Ghrelin protein was identified from day 10 post-partum in the wallaby, much earlier than the onset of its production in humans and rats, suggesting that it is either necessary for stimulating appetite at this early age or that it is a necessary requirement for a functional stomach. Circulating ghrelin and pituitary ghrelin receptor expression were low during early pouch life in the tammar, and increased to a peak at approximately day 70-120 post-partum. Ghrelin stimulates GH release from midgestation in humans when pituitary ghrelin receptors are present and responsive to ghrelin in vitro (Shinar et al., 1998). Since tammars had heightened plasma ghrelin concentrations, pituitary ghrelin receptor and GH expression in vivo it is possible that ghrelin may be stimulating GH release for at least part of development. However, given that elevated GH levels during early life do not appear to be important for normal growth to term, future studies should focus on other possible roles of GH during development such as growth and differentiation of the pituitary gland itself. In
addition, since ghrelin knockout mice do not have an obvious growth or appetite disruption, further studies may determine whether absence of ghrelin or its receptor in the pituitary affect GH production and pituitary morphology.

**GROWTH AXIS GENES**

The genes that control growth of the somatic tissues are highly conserved in vertebrates, both in their structural makeup and in the timing of their expression. IGF-1 and 2 are equally important in embryonic and fetal growth (Baker et al., 1993). However, IGF-2 is not generally considered to be an endocrine growth factor during development and most of its actions are considered to be autocrine or paracrine. IGF-2 is also an important growth factor produced by the placenta, and in all mammals so far studied is maternally imprinted (paternally expressed; Reik et al., 2003; Suzuki et al., 2005).

While the ontogeny of hepatic *IGF-1* expression is well documented and positively correlated with bodyweight and hepatic GH-Rs, hepatic *IGF-2* expression has not been thoroughly investigated (Bennett et al., 1983; Gluckman, 1984; Glasscock et al., 1990). Hypophysectomy of the full-term sheep fetus causes a decline in plasma IGF-1 but not IGF-2, which confirms that IGF-2 is not under hypothalamic control (Mesiano et al., 1989). In contrast to IGF-1, hepatic *IGF-2* expression is more sensitive to changes in plasma cortisol (Li et al., 1998), of which, increases in late gestation are thought to be responsible for eventual IGF-2 decline. In eutherian mammals, increases in cortisol associated with parturition are thought to bring about maturation of the endocrine growth axis especially in relation to the expression of a mature *GH-R* transcript as has been demonstrated in sheep (Li et al., 1999). However, it is unknown whether similar changes in cortisol affect maturation of the marsupial growth axis and indeed whether these changes are necessary for normal growth axis function.

In the tammar, IGF-1 had a similar pattern of expression and plasma concentration to that of eutherian species, where it is strongly correlated with increases in body weight and *GH-R* expression during development, but more importantly, *IGF-2* expression also increased with post-natal age which suggests that the liver becomes the main source of circulating IGF-2 during development with expression declining prior to
GH-regulated growth. This demonstrates a conserved role of IGF-2 in marsupial growth and development. However, given the altricial nature of marsupial young, the growth effects of IGF-2 would occur during pre and post-natal life.

Unexpected sexual dimorphisms were identified in this study in the expression of *IGF-2* and *IGFBP-3* during development in the tammar. The time of this sex difference coincided with the major period of sexually dimorphic phallus growth in this species (Leihy et al., 2004). There is now mounting evidence that the hormones and growth factors of the GH-IGF axis could play an important role in phallus growth and development. Men with congenital GH-deficiency have a small penis which can be corrected with GH or IGF-1 treatment (Levy and Hussman, 1996; Laron and Klinger, 1998). However, once IGF-1 treatment ceases, the growth achieved regresses. IGF-1 stimulates proliferation of penile smooth muscle cells in the rat, while IGF-1 and IGF-1R expression increases post-natally in the rat (Liu et al., 2001) at the same time that male phallus growth occurs. Both these lines of evidence suggest that the IGF-1R is important for phallus growth and differentiation. It is possible that sexually dimorphic *IGF-2* and *IGFBP-3* during the period of phallus growth may be a conserved mechanism by which all mammals achieve sexually dimorphic phallus growth. To test this, IGF-2 inhibitors could be given orally to tammar pouch young during the critical period of phallus growth and development (day 60-150 post-partum), or recombinant IGF-2 could be given to female tammars during this period to see if this stimulates the female phallus to grow or differentiate. Additionally, quantitation of IGF-2 and IGFBP-3 in the plasma of developing tammars would be needed to confirm that the expression differences translate into protein differences during this period. Reciprocal experiments in eutherian mammals would be necessary to determine how conserved this process is in mammals. However, understanding the role of IGF-2 and IGFBP-3 in growth and differentiation of the phallus may require the production of mice with a conditional knockout of *IGF-1R* expression in the phallus.

The other alternative is that androgens such as testosterone may stimulate the production of IGF-1 and IGFBP-3 via response elements upstream of these genes. However, these have not been investigated or reported so far for any species.
GENERAL CONCLUSIONS

Marsupials have traded the umbilical cord for the teat (Renfree, 1983). Thus, the major period of nutritional exchange in the developing young is during fetal life in eutherians and after birth in marsupials. While some marsupials develop in a pouch, others cling to an external groove or rudimentary pocket where the remainder of development occurs until independence (Tyndale-Biscoe, 1973; Tyndale-Biscoe and Renfree, 1987).

![Figure 36. Timing of GH-regulated IGF-1 in the developing tammar wallaby relative to other developmental events.](image)

GH-regulated IGF-1 appears to develop between 150 and 200 days post-partum in the tammar and not during the perinatal period as occurs in eutherian mammals. This strongly suggests it is a process regulated by developmental stage probably in preparation for nutritional independence of the young (Developmental data from Tyndale-Biscoe and Janssens, 1988). Phase 1 of lactation in which the mammary gland is primed for milk secretion occurs during pregnancy.

Therefore, the suggestion that applies to eutherian mammals that the uterus imposes a restriction on growth axis maturation is not likely to be a consideration during marsupial development. In the tammar, development of the endocrine growth axis occurs gradually over the course of an extended lactation period in contrast to the peri-natal period of development in eutherian mammals. This gradual development of
GH-regulated growth represents a mechanism by which the young can develop independence. Thus, study of the developing marsupial has provided new information on the conserved nature of the process that allows the young mammal to become independent of the mother and establish their own homeostasis relative to available resources and as dictated by the differing reproductive strategies adopted by the two groups of extant therian mammals.