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How might marbling begin?

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Abstract. Marbling is an important meat quality trait, in that it contributes directly to the value of beef on international markets. The development of marbling is not well understood, though there have been some significant recent discoveries regarding adipogenesis in general. This article describes a working hypothesis around the early events of marbling. It attempts to rationalise findings from several mammalian experimental systems on hyperplastic growth of adipocyte precursor cells.

Introduction

The term 'marbling' refers to the appearance of white flecks or streaks of adipose tissue between the bundles of muscle fibres in bovine skeletal muscle (Harper *et al*. 2001). While the trait is of most interest in muscles with relatively high commercial value (striploin, *longissimus dorsi*), it is also expressed in other skeletal muscles, though to different extents (Brackebusch *et al*. 1991). Sheep, pigs (Nurnberg *et al*. 1998) and some experimental mouse lines (Kawaguchi *et al*. 2002) express a similar trait. Intramuscular fat deposition is also observed in humans of advanced age, or as part of particular disease processes (Kirkland *et al*. 2002), though this does not necessarily imply shared mechanism of deposition.

Marbling in cattle can be measured ultrasonically in the living animal (W. Upton and M. Woolcott, unpublished data) and visually in the carcass (Tume 2004). Marbling fat scatters ultrasound waves and, hence, appears as bright regions against a dark background in the images of *longissimus dorsi* muscle. Some subjective judgement is required to differentiate marbling fat from connective tissue. In the carcass, it is visually scored at one anatomical position and boundaries have been set to discriminate it from other fat depots. That is, marbling fat does not include fat that forms a connection with any of the subcutaneous or intermuscular fat depots observed at the carcass quartering site. When connections exist, the seam of fat is scored as an 'ingression of fat into the muscle' and excluded from the marbling score. From a developmental point of view, this discrimination may not be valid, as both depots are likely to consist of fat cells (adipocytes), connective tissue and blood vessels. There is considerable variation in the distribution of marbling fat between individual animals, even when assessed at one anatomical site. At one extreme there are the fine, evenly dispersed flecks or streaks of white fat (shimofori, or snow flake marbling). At the other extreme there are thick, coarse channels of fat that merge into the intermuscular fat depots. Again, once the carcass is sufficiently cold for the fats to become opaque, regions of higher connective tissue are not easily discriminated from regions of higher fat content.

Under the microscope, marbling fat is a true adipose tissue, in that it is comprised of adipocytes embedded in a connective tissue matrix in close proximity to a blood capillary network. Mature marbling adipocytes are roughly spherical cells, with diameters of 40–90 μm. The cells may be smaller, on average, than adipocytes from other fat depots of the animal (Cianzio *et al*. 1985; May *et al*. 1994; Lee *et al*. 2000), though the size of the cells varies widely (G. Harper *et al*. unpublished data). At this time, it is not clear if the cellular size difference reflects features of the muscle environment within which these cells develop, or a genetically determined growth potential of the cells.

Marbling adipocytes normally appear in clusters or 'islands'. These islands become visible macroscopically when they contain between 10 and 15 cells. When viewed histologically (Fig. 1), it is possible to find adipocyte islands containing many hundreds of cells, grouped around well-developed capillary beds.

While muscle cells do store some fat in droplets, the naked eye would not detect such stored fat in chilled bovine carcasses. Hence, it is not regarded as a major contributor to the marbling trait, though it certainly is a contributor to the 'intramuscular fat %' trait.

What, then, is the source of the cells that subsequently develop into marbling within meat?

Muscle stem cells with adipogenic potential

Stem, or progenitor, cells are defined by their capacity for self-renewal and subsequent capacity to differentiate into cell types with overtly specialised form and function. Pluripotent stem cells have the capacity to differentiate into

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various cell types with diverse function. For example, under appropriate conditions *in vitro*, stem cells have been shown to differentiate into muscle, cartilage, bone or neural cells (Lagasse *et al*. 2001). Researchers have used specific molecular markers and transplantation studies to demonstrate that stem cells exist within mammalian skeletal muscle (Grounds 1999; Seale *et al*. 2001; Asakura 2003), though the proportion of these cells is likely to be very low compared with the other cell types. In adult bovine skeletal muscle, cell types include: myocytes (multinucleated muscle cells which perform the contractive work of muscle and form the major textural mass of meat); fibroblasts (which form the connective tissue that binds parallel muscle fibre bundles and forms the gristle within meat); endothelial cells (which surround the blood vessels); macrophages (which have a role in clearing the tissue of cellular debris); mast cells (which have a role in inflammation); and adipocytes (which store fat and influence development of other adipocytes). The relatively minor pool of stem cells is believed to be maintained by asymmetric division. This is a process whereby cells divide into 2 daughter cells (as in mitotic division), but where the 2 daughters are not identical in terms of their destiny; one remains a stem cell or equivalent, while the other differentiates into a cell of specialised function (Rao and Mattson 2001). One function of the stem cells is to provide precursor cells for repair and regeneration of skeletal muscle and its supportive structures (Grounds 1999).

The stem cells within skeletal muscle can be subdivided into classes, based on cell surface protein markers and experimental dye efflux properties. One class is the muscle satellite cells, from which are derived the myogenic precursor cells which function in postnatal tissue growth and regeneration (Grounds 1999). Other classes include the 'side populations' that are defined by exclusion of the Hoechst 33342 dye, as well as differential expression of such cell surface markers as Sca-1 and CD45. The importance of these experimental cellular phenotypes is, first, that they enable a fluorescence-activated cell sorter to separate and enrich different cellular populations for independent study. Second, these phenotypes provide a clue to the origin of each of the cellular populations found in muscle (myocytes, adipocytes, fibroblasts etc.).

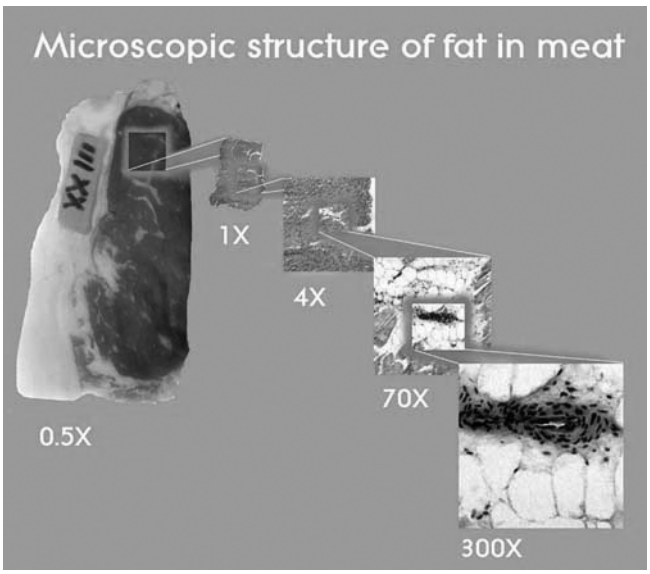

Figure 1. Histological images of marbling fat in muscle, showing the structure of marbling fat at magnifications of 0.5×, 1×, 4×, 70× and 300× (source: P. G. Allingham). Images demonstrate the distribution of adipocytes in the connective tissue seams and around capillaries. Composite image has been reduced about 5-fold, for presentation.

Once committed to a myogenic fate, muscle satellite cells migrate into a space between the basement membrane and the sarcolemma of the myocyte, closely associated with the muscle fibre bundles (Asakura 2003). Here they are believed to remain mitotically quiescent, until induced to proliferate or, ultimately, to differentiate into multinucleated myotubes, following a developmental programme or in response to environmental stimuli (Beauchamp *et al*. 2000; Seale and Rudnicki 2000). The ratio of quiescent satellite cells to myofibre nuclei remains relatively constant over several cycles of degeneration and regeneration. This supports a view that their numbers are maintained by asymmetric division (Asakura 2003). The fact that satellite cells express some specific markers for myogenic cells (e.g. M-cadherin, Pax7 and Myf5; Beauchamp *et al*. 2000, Seale *et al*. 2000 and Seale and Rudnicki 2000), suggests that they are already committed to a myogenic fate, once appropriate stimuli are provided. Nonetheless, recent experiments have suggested that the cells retain the capacity to differentiate into other cell types.

Asakura *et al*. (2001) and Wada *et al*. (2002) found that muscle satellite cells can differentiate into adipocytes or osteocytes, when isolated and grown in culture. Asakura *et al*. (2001) isolated satellite cells from adult mouse skeletal muscle and grew them in culture, to form what they thought were myogenic precursor cells or myoblasts. When these cells were exposed to a series of adipogenic stimuli (methyl-isobutylxanthine, dexamethasone, indomethacin and insulin) they adopted a rounded morphology and accumulated lipid in cytoplasmic vesicles; both characteristic features of adipogenesis. In another experiment, they isolated individual muscle fibres and maintained them in organ culture with Matrigel. This is a commercial basement membrane extract that mimics the extracellular matrix environment within organs, without specifically inducing adipogenesis. Isolated fibres have satellite cells attached and, hence, constitute an experimental system that more closely approximates the environment in which satellite cells normally grow. Again, the adipogenic stimulators induced satellite cells to differentiate into

adipocytes, as shown morphologically and by expression of an early gene marker for adipogenesis (PPARγ2).

Wada *et al*. (2002) isolated individual satellite cells and cultured them clonally. They showed that the clones alone were sufficient to produce adipocytes when exposed to appropriate culture conditions (added γ-linolenic acid), though the clones would not spontaneously develop into adipocytes. These authors also found that the pluripotent muscle stem cells expressed some of the protein markers for myogenesis (MyoD), adipogenesis (PPARγ) and osteogenesis (Runx2) simultaneously. They developed a 'stock options' model for determination of cell fate, whereby pluripotent stem cells present in muscle keep their functional options open, until external and local stimuli drive them towards some particular lineage. The clear implication of these findings for our understanding of marbling in cattle, is that various uncommitted stem cells within skeletal muscle could develop into marbling adipocytes, not just preadipocytes. The questions then become: 'Do all pluripotent stem cells within muscle come only from muscle?'; and 'What induces these to differentiate in bovine muscle?'.

There is other strong evidence that mammalian muscle contains stem cells. Transplantation studies, using lethally irradiated mice, have shown that muscle extracts contain all the pluripotent stem cells required to completely reconstitute the haematopoietic repertoire of the animal, just as bone marrow extracts do (Geiger *et al*. 2002; Gussoni *et al*. 1999; Issarachai *et al*. 2002; Jackson *et al*. 1999; Kawada and Ogawa 2001; Pang 2000). These researchers showed that muscle contains haematopoietic stem cells, in addition to other stem cell populations. A concensus has developed that these cells are enriched in the 'side population' of cells prepared by fluorescence-activated cell sorting (Asakura 2003) and that they are a separate population from the satellite cells.

As Asakura (2003) describes, studies in wild-type and knock-out mice (e.g. *Pax7* –/–) have suggested that some of the side population of stem cells within muscle could enter the tissue from the blood stream. Some of the cells exhibit the surface antigen CD45 which is known to be associated with the haematopoietic lineage (Asakura 2003). Further, it is clear that some stem cells derived from bone marrow can differentiate into muscle satellite cells, but it cannot be said that all satellite cells are derived from bone marrow. What is clear, is that the intramuscular environment exerts some influence on the differentiation of stem cells, whether or not they have come from the bone marrow. This environmental effect is likely to relate, at least, to physical forces (repeated compression–stretch), blood supply, extracellular matrix macromolecules and growth factors. So environment and the morphological and functional plasticity of muscle stem cells come together to determine the fate of these cells.

Other pluripotent cells have also been isolated from muscle and these may play a role in initiation of marbling. De Angelis *et al*. (1999) and Minasi *et al*. (2002) have described stem cells that appear in close association with the vasculature. Young *et al*. (2001) have described mesenchymal-like stem cells derived from muscle connective tissue. The latter cells are particularly attractive as a progenitor for marbling, because we know marbling develops within connective tissue seams and in close proximity to capilliary beds.

It is reasonable to hypothesise that: (i) several forms of pluripotent stem cells within muscle could be the progenitors of marbling adipocytes; and (ii) this pool of stem cells may be replenished, from time to time, with cells from other parts of the body, such as the bone marrow (Fig. 2; Issarachai *et al*. 2002). The size of this pool of stem cells will also be influenced by the rate of differentiation to cells into other lineages (e.g myogenic) and the rate of loss due to cell death.

Once intramuscular adipogenesis has begun, we hypothesise that it follows a similar series of events to adipogenesis in other depots, independent of the environmental inputs or constraints. If this were the case, then we would expect several events, both molecular and structural to ensue, as shown in Figure 2 (Ailhaud *et al*. 1992; Smas and Sul 1995; Gregoire *et al*. 1998; Kirkland *et al*. 2002).

As part of this scheme, we describe a 'preadipocyte'. This is an intermediate form, through which a cell develops before expression of differentiated adipocyte characteristics. Our current concept is that such a cell type cannot be characterised as a separate or reproducible entity. A view that is more strongly supported, is one that regards the pluripotent stem cells as a flexible population of cells that have the capacity to express primordial features of several differentiated cells simultaneously, but are yet to be committed to any particular lineage (Asakura 2003). Once the pluripotent stem cells become committed to the adipogenic lineage, they develop inexorably and terminally towards that form. The hypothesis that preadipocytes might exist in muscle as a specific intermediate cell type arose from *in vitro* studies using long-lived cell lines such as 3T3-L1, which reproducibly undergoes adipogenesis in culture (Wang *et al.* 1994).

Once differentiation begins, preadipocytes undergo characteristic changes. Early changes include: reduced collagen synthesis; changes in the types of cell-matrix adhesion molecules; and changes in the intracellular proteins responsible for cellular size and shape (Smas and Sul 1995). Synthesis of the Delta-like protein (DLK) is significantly reduced, suggesting that it may play a role in maintenance of the preadipocyte stage and also providing a technical opportunity to identify preadipocytes (Smas and Sul 1995).

Studies in culture have found that cell cycle arrest is a prerequisite for differentiation of adipocytes. Cells that have

progressed down the differentiation path towards adipocytes, can no longer divide. Growth from this point is focused on deposition of intracellular fat globules. A great deal of effort has been expended on characterisation of the biochemical and hormonal stimuli that drive preadipocytes to differentiate in culture (Smas and Sul 1995). There is little known about initiators or stimulators of adipogenesis within muscle, but the extent of our knowledge is highlighted below.

Developmental triggers

Our view is that pluripotent stem cells lie dormant within the tissue until some external stimulus induces them to differentiate. An alternative view would be that some stimulus might induce recruitment of pluripotent stem cells from the blood stream into the muscle, where they supplement the pool of stem cells already present and lead on to a change in cellularity of the tissue. Certainly, stem cells from the blood are now believed to supplement the repair capacity of muscle (Grounds 1999). So what developmental cues might increase the number of stem cells within the growing muscle, which might then develop into marbling? There are a number of animal or environmental characteristics that are known to influence the expression of marbling, though this may not result specifically from hyperplastic growth of pluripotent stem cells.

Normal ageing

Age is a major determinant of adiposity in mammals, with the developmental programmes of growth, puberty and ageing driving significant changes in fat distribution and total fatness (Vernon 1981, 1986; Kirkland *et al*. 2002). As pointed out by Pethick *et al*. (2004), marbling and the closely related trait of intramuscular fat % are late developing traits, though the prerequisite adiposity of muscle develops significantly earlier than macroscopic expression of the trait (Wegner *et al*. 1998). Mammals of advanced age show redistribution of fat into non-adipose tissues and, specifically, into skeletal muscle (Kirkland *et al*. 2002). The fact that finished cattle and old age humans both show intramuscular fat deposition is interesting. It may suggest common developmental mechanisms, though it is unlikely to imply that finished and marbled cattle are on the edge of senescence. In the Wagyu production system of Japan, animals with a genetic disposition to marble are weaned at ages of 4–6 months and are subsequently fed on high energy diets for ~20 months. This means they are less than 3 years old at the time they are expressing the trait. Likewise,

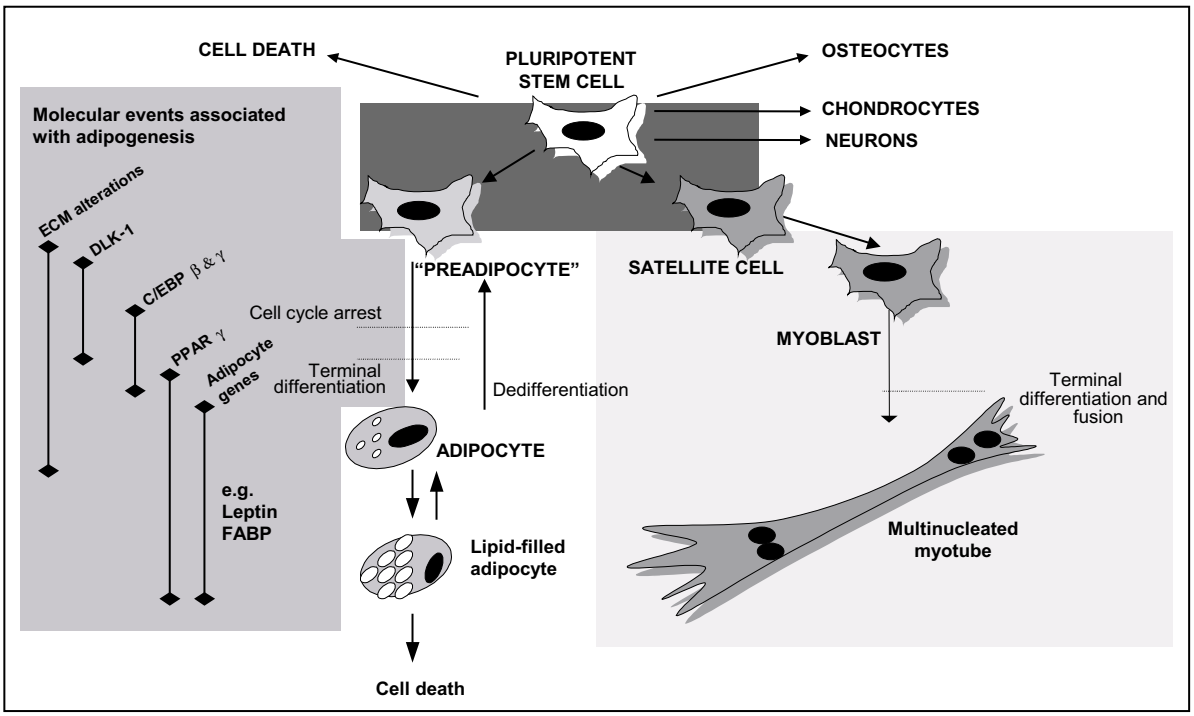

Figure 2. Schematic diagram representing the development of mature adipocytes from pluripotent stem cells in the intramuscular environment. Dark grey area represents some fluidity in the stem cells, in terms of form, function and differentiation destiny. Grey shaded areas indicate the environmental characteristics specific to the perimysial environment. Some molecular events known to accompany adipogenic differentiation are shown on the left; the line position and length indicate periods of elevated gene expression. 'ECM alterations' are changes in turnover of existing and newly synthesised extracellular matrix components, particularly through the action of matrix metalloproteinases. DLK-1, C/EBP and PPARγ are standard abbreviations for genes involved in adipogenesis (Gregoire *et al*. 1998). 'Adipocyte genes' are a network of genes that are coordinately regulated in terminally differentiated adipocytes.

marbling in Angus cattle is often expressed in animals that are less than 28 months old (Hocquette *et al*. 2003); certainly not equivalent ages to old age in humans.

Histological studies show that ruminant fat depots, including intramuscular fat, continue to expand with age, unlike bone and muscle, which both reach developmental maxima during adult life (Hood and Allen 1973). Likewise, the capillary networks of muscle continue to develop (Crandall *et al*. 1997). These provide a rich source of haematopoietic and other stem cells, as well as a nutrient supply for developing preadipocytes. Recent studies by Hocquette *et al.* (2003), with muscles from 3 different breeds of cattle, suggest a close relationship between intramuscular fat % and the aerobic–anaerobic metabolism of the muscle as judged by enzymatic pattern (R^2 = 0.57 for cytochrome c oxidase content *v.* muscle triacylglycerol). This is consistent with developmental changes in tissue oxidative state and, perhaps, capillarity, because cytochrome c oxidase is an oxidative marker.

Adipocytes that are already present, for example at 12 months of age, presumably continue to grow in diameter (Hood and Allen 1973) as they deposit fat and are joined by new adipocytes (Leibel *et al*. 1989). While there is likely to be some ongoing loss of adipocytes due to cell death, this death rate has not been measured in cattle. For comparison, the lifetime of a rat adipocyte is believed to be ~140 days. An implication of this relatively slow turnover rate, is that early life events are likely to play a role in determining the number and size of adipocytes at some later stage of life (Caserta *et al*. 2001; Kirkland and Dobson 1997; Klyde and Hirsch 1979).

Muscles vary in the chronology of intramuscular fat development. Cianzio *et al*. (1985) have shown that adipocytes appear earlier in the *longissimus dorsi* muscle than the *pectoralis*, for example. More recent studies have suggested that adipocytes of different sizes and locations express different genes and biochemical constituents (Lee *et al*. 1997).

Age is clearly an important parameter in fat depot development. Recent studies in other species show that advancing age does not influence all stem cell populations equally. Germ line stem cells, for example, gradually decline in number and this is a precursor to menopause (Schesslinger and Van Zant 2001). In contrast, haematopoietic stem cells are present in the marrow through to old age and do not necessarily decrease as a function of age. This means the potential for marbling precursor cells to be presented to the tissue for subsequent adipogenesis remains throughout adult life. Expression of C/EBPα, an important driver of adipogenesis, declines substantially with age in rats. This means that older animals are less able to support adipogenesis, even though the animal might have a genetic predisposition to fatness and a high-energy diet conducive to fatness. The expression of other transcriptional factors that are essential to adipogenesis is also reduced with advancing age, while the expression of at least one inhibitory transcriptional factor (C/EBPβ-LIP) is increased. These factors work together to lead to a state of 'dysdifferentiation', defined by Kirkland *et al*. (2002), whereby cells that would normally differentiate into adipocytes (such as preadipocytes, mesenchymal stem cells or even satellite cells) accumulate in partially differentiated forms in the fat depot. These authors refer to these cells as mesenchymal adipocyte-like default (MAD) cells, because they exhibit some of the characteristics of adipocytes (lipid droplet accumulation, rounded shape), yet they appear in tissues that do not normally accumulate adipocytes (muscle, bone marrow). This latter mechanism seems reasonable in the context of marbling development, though such an hypothesis might imply acceleration of the mechanisms normally operating in humans of advanced age.

Vitamin A status

Low dietary vitamin A levels may stimulate the commitment of multipotent stem cells to the adipocyte lineage. Oka *et al.* (1998*a*, 1998*b*) and others (Naruse *et al*. 1994) have shown that a reduction in plasma vitamin A levels tends to increase the marbling score at slaughter. This was confirmed by showing that vitamin A supplementation at specific ages can reduce the subsequent marbling score. Recent work by our group has demonstrated the effect of vitamin A status in another breed of cattle (Australian Angus). This suggests the generality of the finding amongst cattle, other than the Japanese Black breed, that share a genetic propensity to marble (Kruk *et al*. 2004). Indeed, the effect has also been observed in genetically lean pigs (D'Souza *et al*. 2003).

The hypothesis that low dietary vitamin A (or more correctly carotenoids) may have a role in the initiation of marbling is supported by studies with adipogenesis in other species and *in vitro* (Gregoire *et al*. 1998; D'Souza *et al*. 2003). The vitamin A axis has been functionally linked to the thyroid hormone axis (T_3 and T_4) and the insulin-like growth factors (IGF's). Interestingly, Oka *et al*. (1998*b*) investigated T_3, T_4, insulin and IGF-1 levels in Japanese Black cattle and concluded that low vitamin A status significantly changed the normal plasma levels of these hormones. These hormonal axes may work together when recruiting pluripotent stem cells into muscle or determining the number of preadipocytes that differentiate. Furthermore, Oka *et al*. (1998*b*) demonstrated an interesting age-dependent sensitivity of cattle to the effects of vitamin A. This is unlikely to be due to a functional depletion of stem cell populations, given the finding that tissue stem cells persist even in senescent mammals and geriatric humans (Young *et al*. 2001; Schlessinger and Van Zant 2001). Japanese Black cattle, or other breeds for that matter, are unlikely to be senescent at the time they deposit marbling most rapidly.

Gender effects

Many experiments have examined the marbling levels in the different gender classes of cattle (heifers, cows, bulls and steers). Table 1 summarises many of the results. A general conclusion would be that, for a given slaughter weight and time on feed, heifers have higher marbling levels than steers. Steers, in turn, have higher marbling levels than bulls. In more extensive studies incorporating a serial slaughter, however, no differences were identified (Fig. 3). Indeed, when intramuscular fat % was expressed relative to total fatness, steers had marginally higher intramuscular fat % than heifers. In Charolais heifers, those which had received an ovarian tissue implant had higher marbling levels than control animals (Lunt *et al*. 1990). The age of castration may also have an effect on marbling level. Meaker *et al*. (1986) found that calves castrated at birth had higher marbling levels than those castrated at 6 months. Worrell *et al*. (1987) found that castration at 70 days of age, compared with 230 days, also increased marbling levels. These observations suggest that the sex hormones influence growth and development of intramuscular adipocytes, though the mechanism is not defined yet.

Candidate genes and their relationship to adipogenesis

Most studies support the conclusion that multiple genes influence marbling or, indeed, carcass fatness in cattle (cf. Morton and Lio 1997). Moreover, the effects of each gene are small, relative to total variation in the trait. Nonetheless, it is possible to locate regions on a chromosome (quantitative trait loci, or QTL) which are associated with differences in the marbling phenotype and, perhaps, to later identify the genes which confer marbling capacity. The search for, and identification of, genes with specific effects on marbling have been discussed by Barendse *et al*. (2004).

A major gene known to affect carcass fatness is the gene responsible for double muscling in cattle: GDF8 (myostatin). The gene not only affects the size of the muscle that develops, but also the proportion of connective tissue within the muscle and intramuscular fat % (G. S. Harper, P. G. Allingham, M. E. McKay unpublished data). Given the site of development of marbling, it seems likely that these 3 observations are mechanistically linked. The mutations in the GDF8 gene have been described by Grobet *et al*. (1997) and Kambadur *et al*. (1997). The GDF8 gene product is a growth regulator for muscle development. Mutations that affect its function generally result in increased muscle mass (McPherron *et al*. 1997). In cattle, these mutations also cause a decrease in the deposition of fat tissues and changes in the conformation of the skeleton (Hanset *et al*. 1982; Shahin and Berg 1985). With respect to development of marbling fat, Wegner *et al*. (1998) demonstrated that GDF8 mutant double-muscled animals have: (i) fewer islands of adipocytes in their *longissimus dorsi* muscle; (ii) slower growth of these islands; and (iii) smaller adipocytes in marbling islands than wild-type cattle.

For the minor genes affecting marbling, there is evidence for at least 5 QTL of moderate effect. Genes underlying 2 of

Table 1. Relative influence of animal class on marbling levels

Animal class with the highest marbling level in each study is indicated by '1'; animal classes with the next highest marbling levels in each study are indicated by '2' and then '3'

Animal class				Study
Cows	Heifers	Steers	Bulls	
		1	2	Shackleford *et al*. (1992)
		1	2	Huerta-Leidenz *et al*. (1991)
1	2			Vincent *et al*. (1991)
	1	2	3	Jones *et al*. (1990)
		1	2	Johnson *et al*. (1988)
		1	2	Jones *et al*. (1986)
	1	2		Slanger *et al*. (1985)
		1	2	Ockerman *et al*. (1984)

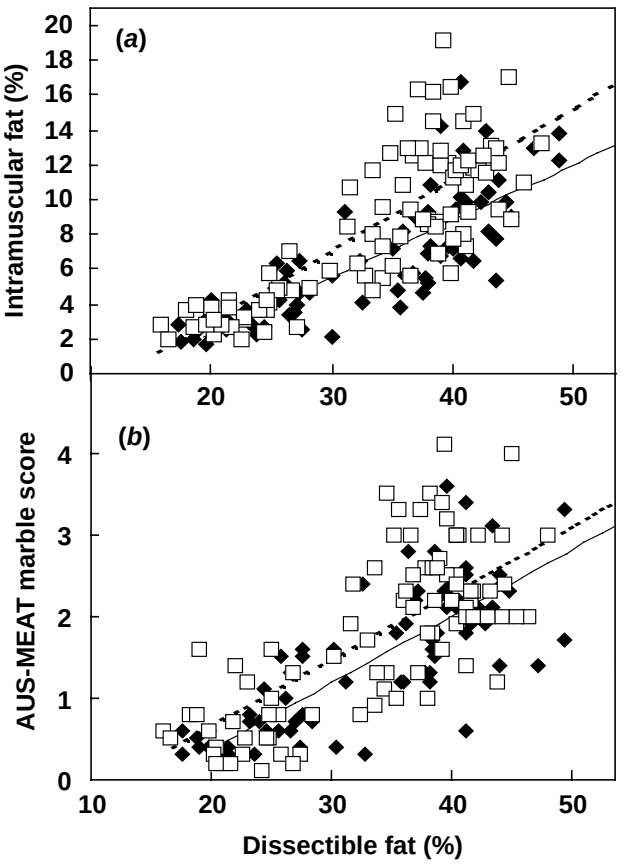

Figure 3. Relationship between dissectible fat (%) and (*a*) intramuscular fat % and (*b*) AUS-MEAT Marble Score, for steers (□) and heifers (◆). In (*a*), for heifers, $y = -4.07 + 0.32x$ and for steers, $y = -5.25 + 0.40x$ ($R^2 = 0.658$, $P<0.0001$). In (*b*), for heifers, $y = -1.248 + 0.081x$ and for steers, $y = -0.970 + 0.081x$ ($R^2 = 0.566$, $P<0.0001$) (A. K. Pugh *et al.* unpublished data).

these confirmed QTL are of most interest, in terms of understanding the mechanism of marbling. Barendse *et al*. (2004) found that polymorphisms near the thyroglobulin gene (TG) on chromosome 14 are associated with marbling capacity. The TG gene spans 300 kb of DNA (Mercken *et al*. 1985) and encodes a protein that, indirectly, plays a significant role in regulation of metabolic rate. The polymorphisms that are associated with variation in marbling are not within the coding region of the TG gene, but lie within the 5′ untranslated region of the gene, which may be involved in regulation of the gene's activity.

In response to thyroid stimulating hormone, the TG gene is expressed in the thyroid epithelia cell (Salvatore *et al*. 1980). The TG protein is secreted into the lumen of the thyroid gland. At the same time, and under similar hormonal regulation, iodide anion is actively transported into the lumen and activated into a form that can react with the tyrosine amino acid residues of the TG protein. Monoiodotyrosine, diiodotyrosine, T_3 and T_4 are produced through the action of transiodinase (Salvatore *et al*. 1980). The modified TG protein is then taken up by the thyroid epithelial cell, by receptor-mediated endocytosis, then the iodinated tyrosine residues are liberated within the acidic environment of the lysosome (Tietze *et al*. 1989). Monoiodinated and diiodinated tyrosine are recycled within the thyroid gland, because iodine is a scarce nutrient. The final active endocrine products, T_3 and T_4, are then transported across the thyroid epithelia cell, then released into the blood stream and on to the sites of action.

Thyroglobulin by itself has an indirect role in metabolic regulation, being a protein intermediate in the production of the T_3 and T_4 hormones. Nontheless, genetic variation in the rate or timing of TG gene expression could subtly influence the release of the thyroid hormones, T_3 and T_4. Evidence in support of this hypothesis comes from a number of sources. First, levels of the thyroid hormones have been implicated in the development of adipocytes in muscle (Salter 1950) and the differentiation of adipocytes *in vitro* (Ailhaud *et al*. 1992; Smas and Sul 1995). They are also implicated in metabolic rate with high levels of thyroid hormone associated with high metabolic rates. In the context of marbling, it would appear more likely to us that lower levels of the thyroid hormones would be more consistent with high marbling, since this state would lead to lower metabolic rate. In other words, we would expect more energy available within the muscle for deposition as fat within the tissue. There is currently no direct evidence for effects of the thyroid hormones on stem cell numbers within the muscle.

The second confirmed marbling QTL is on chromosome 5. The closest marker is CSSM34, which is close genetically to the gene RARG (retinoic acid receptor gamma; Barendse 1997). Again, the polymorphism associated with marbling is likely to lie within the non-coding sequence of the RARG gene. The RARG gene product is involved in regulation of transcription of a large family of genes. All-trans retinoic acid, one of the retinoid family of compounds, binds to RARG which, in turn, binds to specific sequences of the DNA in the nucleus. RARG binding results in an increase in the rate of transcription from the gene to which it bound. The retinoic acid receptors (RAR) and the retinoid-X receptors (RXR) are important regulators of normal development of organs and tissues (Solomin *et al*. 1998). While the detailed mechanism is not known, it is interesting to note the relationship between low vitamin A status and high marbling score that was mentioned earlier.

The discovery of these QTL implicates genetic variation, in either the thyroid hormone or retinoid receptor axes, in individual differences in marbling capacity. Still, the location of QTL near coding sequences is not proof of their involvement. Three facts council us to be cautious about attributing cause and effect. First, the genome is rich in coding sequences, with ~30 000 expected in cattle. There may be alternative candidates from other pathways or, as the Beef Quality CRC cross breeding data (Newman and Reverter 2000) suggests, there may be no major genes involved, at least in the British and *Bos indicus* breeds of cattle studied. Moreover, due to the relatively small size of the effects of those QTL identified to date, it is not feasible to use gene transfer experiments to prove that the target sequence actually does cause the effect. Third, if the QTL is not the gene itself, it may be the result of several favourable genes located near each other in a complex held together by linkage disequilibrium. Such a complex is not stable, since linkage disequilibrium would decay through the normal processes of recombination. Hence, without specifically recognising that a complex of genes might be involved, it would be difficult to breed specifically for the effect.

Muscle pathology

It is clear that fat accumulation in muscle can occur in mammals other than cattle, but only under relatively extreme pathological conditions in most species. Fat in muscle of Duroc pigs and sheep for example, can be as high as 10% (w/w). The expression of the marbling phenotype in cattle is encouraged through long feeding strategies and high-energy diets. Such feeding regimens are generally not used in sheep or pigs. It is our opinion, therefore, that marbling in most cattle does not result from development of a disorder or disease process. This may not be true for the extremes of marbling, such as seen occasionally in the Japanese Black breed of cattle (S. Tsuji, pers. comm.). It seems more likely that accumulation of intramuscular fat is related to an accumulation of MAD cells and that this accumulation is enhanced by genetic predisposition, provision of a high energy diet relatively early in life and, perhaps, other features of the feedlot environment, such as inactivity.

Industrial implications

Irrespective of the mechanisms of marbling, there will continue to be industrial interest in its developmental biology. While we doubt that 100% of the population variance in marbling will ever be accounted for, we confidently expect that research will generate sufficient knowledge for the cattle industry to manage marbling in a production setting.

In this context, it seems likely that when marbling is a required commercial end point, producers should focus on initiation of more preadipocytes in muscle of animals that have a genetic predisposition to marble. These cells could then go on to produce more mature adipocytes when the animal is the appropriate age and eating a suitable diet, to express marbling potential more fully. Given the likelihood of a greater number of multipotent stem cells and preadipocytes in younger animals, focusing nutritional treatments on the young animal (less than 200 kg HCW) would seem to be the right approach. Data presented by Pethick *et al*. (2004) support the view that cattle enter the feedlot with a certain intramuscular fat %, which increases arithmetically during finishing. At the cellular level, we interpret this to mean that there is a certain number of adipocytes in muscle at feedlot entry and also that high energy finishing regimens simply fill these cells with lipid. Therefore, a nutritional strategy that targets both increased adipocyte numbers and size, is likely to have most success in increasing marbling score. Despite the indications that vitamin A depletion might initiate more preadipocytes, animal health and welfare issues should discourage the industry from adopting this strategy.

The most efficient path between our current knowledge and the knowledge we need to manage this phenotype requires constant awareness of developments in other streams of mammalian research. Gene function studies and the global chromosomal mapping experiments in humans, mice and rats provide a boundless resource for scientists working in the applied fields and profiting from the phenotypic variation that has occurred naturally in any one species: in our case, the bovine.

What next?

To progress our understanding of marbling beyond this point, these are some of the experiments that need to be undertaken. First, we believe that more of the natural history of marbling needs to be defined. How does marbling develop in cattle that have the genetic disposition for development of the trait? When does it first begin to develop and what are the sources of these cells? Such studies might focus on young animals for example from birth to 200 kg liveweight.

Second, we need to test some of the cellular hypotheses proposed here in relation to the early development of marbling. This might involve some relatively aggressive nutritional treatments, even though these may not be commercially realistic. The long-term outcome could be early life nutritional or growth treatments that influence the subsequent expression of marbling. Examples might include modulation of the thyroid hormone axis, or modulation of the diet during the preweaning period, as in European vealer calf diets.

The power in these experimental approaches will come from a systems biological approach. Researchers will need to investigate the trait at a number of biological scales: DNA polymorphisms; RNA expression; protein synthesis; enzyme activity; cellularity; tissue structure; and visual expression in a chilled carcass. A complex trait like marbling demands such an approach, because no single factor determines a large proportion of the trait variation in the population.

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