Chapter 13
Calcitonin Receptor Expression in Embryonic, Foetal and Adult Tissues: Developmental and Pathophysiological Implications

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Abstract It has been well established that the calcitonin receptor (CTR) mediates the actions of calcitonin in bone homeostatic mechanisms during growth and in adulthood. However, the widespread expression of CTR in embryonic, foetal and adult tissues together with functional studies implicates the activity of CTR in other physiological and pathophysiological events including wound healing, cardiovascular disease and some cancers. The development of high affinity anti-CTR antibodies has helped define the roles of CTR in organogenesis and pathogenesis, and has focused our attention on the roles of precursor cells that express CTR. These CTR-positive cells are featured in foetal development, cardiovascular disease and leukaemia. It is hypothesised that the potential to express CTR is a fundamental property of precursors and progeny of the haematopoietic lineages.

Keywords Calcitonin receptor • haematopoiesis • antibody • foetal development • wound healing • cardiovascular disease • leukaemia

Abbreviations

AGM	Aorta-gonado-mesonephros
ALL	Acute lymphoblastic leukaemia

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AML	Acute myeloblastic leukaemia
AM	Adrenomedullin
CD34	Cluster of differentiation antigen 34
CGRP	Calcitonin gene-related peptide
CLR	Calcitonin receptor-like receptor
CNS	Central nervous system
CT	Calcitonin
hCTR	human calcitonin receptor
CTR-ir	CTR immuno-reactivity
CVD	Cardiovascular disease
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescence activated cell sorting
Gb R	Gamma amino butyric acid receptor
GDNF	glial cell-derived neurotrophic factor
GFAP	Glial fibrilliary acidic protein
GFR	GDNF-family receptor
GPCR	G-protein coupled receptors
HMEC	Human microvessel endothelial cells
IF	Immunofluorescence
IHC	Immunohistochemistry
ISH	*In situ* hybridization
[^{125}I]-sCT	[^{125}I]-iodine labeled salmon calcitonin
MAb	Monoclonal antibody
NFkappaβ	Transcription factor
PAb	Polyclonal antibody
PACAP	Pituitary adenylyl cyclase activating peptide
PAC 1	PACAP selective receptor
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
PTH	Parathyroid hormone
RAMP	Receptor activity modifying protein
RANK	Receptor activator of NFkappaβ
RANKL	RANK ligand
RET	(RE-arranged during Transfection) receptor tyrosine kinase
VEGFR	Vascular epithelial growth factor receptor
VIP	Vasoactive intestinal peptide
VPAC R	VIP/PACAP receptor
WB	Western blot

13.1 Introduction

13.1.1 The Original Assignment of the Endocrine Function of the Calcitonin (CT)/Receptor (CTR) System

It is now approaching 50 years since CT was first described (Copp et al. 1962; Hirsch et al. 1963, 1964; Kumar et al. 1963; Macintyre et al. 1964; Copp 1967) and less than 20 years since the cDNA sequence of the CTR transcript was announced together with the predicted amino acid sequence of the receptor (Lin et al. 1991). The original assignment of the endocrine function of the (thyro) calcitonin/CTR system predicted roles in homeostatic mechanisms that regulate calcium metabolism, including the promotion of calcium excretion from kidney, and the inhibition of osteoclast activity and osteolysis. Recently, investigations with a mouse model with low expression of CTR showed results that are consistent with this function (Davey et al. 2008).

This chapter will focus on the additional roles of CTR in developing embryonic and foetal tissues, adult tissues and pathologies. Here we discuss observations and suggest a link between cellular events in which CTR is expressed in foetal organogenesis, with equivalent pathogenic events in the adult. Consideration of these putative roles and associations of the expression of CTR have been made possible by the development of high affinity anti-CTR antibodies.

13.1.2 A Brief Overview of the Pharmacology of CTR, a G-Protein Coupled Receptor (GPCR)

Family B GPCRs include the seven transmembrane (serpentine) receptors for calcitonin (CTR), for adrenomedulin (AM) and calcitonin gene-related peptide (CGRP) (the latter two ligands utilise the calcitonin receptor-like receptor [CRLR or CLR]), vasoactive intestinal peptide (VIP)/pituitary adenylyl cyclase activating peptide (PACAP) (common receptors VPAC1R, VPAC2R and the PACAP selective receptor PAC1), glucagon, secretin, parathyroid hormone (receptors PTHR1 and PTHR2) and several other peptide hormones (Segre and Goldring 1993). CTR has been shown to couple to second messenger systems including adenylyl cyclase, phospholipase C and inositol phosphates systems (Force et al. 1992; Nussenzveig et al. 1994).

CTR mRNA and splice variants have been sequenced from several species (Lin et al. 1991; Gorn et al. 1992; Albrandt et al. 1993; Kuestner et al. 1994; Yamin et al. 1994; Gorn et al. 1995; Nussenzveig et al. 1995). In humans there are several isoforms of CTR (hCTR), all of which result from gene splicing events from a single CTR gene located on chromosome 7 (Yamin et al. 1994). The best characterized variants are the insert-negative (hCTRa) and insert-plus isoforms (hCTRb), which, in human CTR, includes an insert of 16-amino acid residues towards the

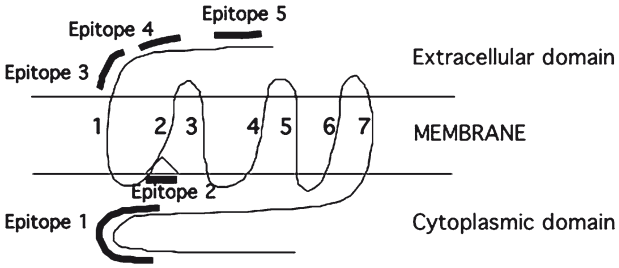


Fig. 13.1 Cartoon of the proposed secondary structure of human CTR, indicating epitopes recognised by specific antibodies

N-terminal domain of the putative second transmembrane span (hydrophobic span II, Fig. 13.1) or intracellular loop 1. The isoform that may be regarded as equivalent in rodent (CTR C1b) includes a sequence of 31-amino acid residues located after span II within the extracellular domain. In either species CTRa is the predominant isoform that is expressed by different subsets of cells within most normal and diseased tissues.

Although the on- and off-rates for CT binding were similar there are some clear differences in the characteristics of the two isoforms when expressed in the BHK cell line. Firstly, hCTRb is significantly impaired in its rate of internalisation (Moore et al. 1995). Secondly, hCTRb displays a significantly reduced capacity to couple to the second messenger enzymes adenylyl cyclase and phospholipase C. Thirdly, stimulation of a transient calcium response was observed only with the hCTRa isoform (Moore et al. 1995).

Furthermore when COS-7 cells were transfected with hCTRa, in contrast to cells transfected with the hCTRb isoform (negative response), the binding of the potent agonist salmon-CT resulted in retardation of the cell cycle with cells stalled in the G_2/M phase (Evdokiou et al. 1999).

13.1.3 CTR and the Formation of Heterodimeric Complexes

Receptor Activity Modifying Proteins (RAMPs) interact with many members of the Family B GPCRs (Sexton et al. 2006) and appear to be generally involved in the cycling of these GPCRs to and from the plasma membrane and their targeting to several cytoplasmic locations depending on the type of RAMP (1, 2 or 3) heterodimer (McLatchie et al. 1998). As the name implies in some instances these accessory proteins modify the active site and hence define the specific ligand. For instance the CGRP receptor is a combination of CLR (CTR-like receptor) and RAMP1.

The expression of RAMPs is far broader in tissues than the range of cell types that express CTR or CLR and therefore it was hypothesized that they may also

dimerize with other GPCRs of Family B. In fact it has been reported that RAMPs form heterodimers with the VPAC1 receptor and PTHR1 and PTHR2 (Christopoulos et al. 2003). In these instances the association of RAMPs did not appear to influence binding but rather internalisation and re-cycling to the plasma membrane.

While the identity of the receptors for CGRP and adrenomedullin (R1 and R2) has been established and are composed of CLR/RAMP 1 and CLR/RAMPs 2 and 3 respectively, the identity of the receptor(s) for amylin, which is a related peptide hormone secreted by pancreatic β-cells, is unclear. Although two pharmacologically distinct forms have been claimed on the basis of studies with some transfected cell lines (Muff et al. 1999; Christopoulos et al. 1999; Hay et al. 2005), the identity of amylin receptors in the context of their true physiological functions has yet to be established (Kikumoto et al. 2003; Wookey et al. 2006).

The classical idea that GPCRs function as monomeric entities has been unsettled by the emerging concept of GPCR homo- and hetero-dimerization (Terrillon and Bouvier 2004). Dimerization is a potential mechanism that could provide high affinity binding sites for the list of CT-like peptides (Foord et al. 2005). A well-established example is the heterodimer formation that is essential, not only for export from the endoplasmic reticulum of the receptor for gamma aminobutyric acid (Gb R1 and Gb R2 (Wookey et al. 2006)), but also for activation of ligand binding at the receptor complex. While it is yet to be established, a further example may include the physiological receptors for amylin, which could be comprised of heterodimers of GPCRs.

13.1.4 The Control of Expression of CTR mRNA

Early studies described modulation of CT binding sites with physiological levels of CT and also glucocorticoids (Wada et al. 1996, 1997, 2001). There are at least seven CTR mRNA transcripts that are variably expressed in different cell types by different species (Moore et al. 1995; Hebden et al. 2000; Anusaksathien et al. 2001). Three promoters are utilised, P1 and P2 in osteoclasts, brain and kidney, whereas P3 is additional and specific for osteoclasts (Hebden et al. 2000; Anusaksathien et al. 2001; Shen et al. 2007). The transcription factor NFkappaβ can be activated by the binding of the ligand RANKL to its receptor RANK. Thus the extracellular RANKL biosythesized by osteoblasts promotes the terminal differentiation of osteoclasts with the induction of multiple associated genes including CTR (Takayanagi et al. 2002; Kim et al. 2006). The transcription factors, SP1 and SP3, have been implicated in transcriptional control (Pondel et al. 2002, 2003).

An intriguing finding was described in relation to imprinting of CTR in mouse (Hoshiya et al. 2003). It was reported that CTR was expressed by the maternal allele in the mouse brain whereas no allelic bias was found in other tissues. Thus the report concluded that CTR was imprinted in a tissue specific manner with predominant expression from the maternal allele in the brain.

13.2 Widespread Expression in Adult Tissues

Since the discovery of CT and the teleost equivalent, salmon CT (sCT), the radioligand [^{125}I]-sCT has been established as a useful high affinity ligand of mammalian CTR for mapping and pharmacological studies of the CT binding site. More recently some techniques of molecular biology, namely PCR and in situ hybridisation (ISH) have also been used in mapping studies of CTR (Table 13.1).

The widespread expression in many tissues may reflect a role for CTR in local events such as changes in the calcium activity in the micro-environment. Such a role for PTHrP/PTHR has been discussed previously (Brown et al. 1996). If this turned out to be the case for CTR then there may also be a role for an uncharacterized CT-like peptide in the tissue micro-environment.

This chapter will focus on the expression of CTR in various embryonic, foetal and adult tissues, with particular reference to hematopoietic and vascular systems, which are involved in important pathophysiological processes including wound healing, atherosclerosis and leukemia.

13.3 Studies with Anti-CTR Antibodies

13.3.1 Anti-CTR Antibodies Used in Early Studies

The generation of anti-CTR polyclonal antibodies against human (Stroop et al. 1995; Nygaard et al. 1997; Perry et al. 1997) and rodent CTR (Perry et al. 1997) using CTR-fusion proteins for immunization has been reported, and have been used in immunoblotting (Stroop et al. 1995; Nygaard et al. 1997; Perry et al. 1997) and visualization of CTR-positive bone cells using IHC (Quinn et al. 1999). Other polyclonal anti-CTR antibodies have also been reported for studies with human bone cells (Tobon-Arroyave et al. 2005; Vered et al. 2006).

13.3.2 The Development of High Affinity Anti-CTR Antibodies

With the recent availability of high affinity anti-CTR antibodies (Tikellis et al. 2003; Tolcos et al. 2003; Becskei et al. 2004; Silvestris et al. 2008; Fukada et al. 2007; Wookey et al. 2008) the detection with increasing precision and definition, and identification of individual cell types that express CTR in normal and diseased tissues has been made possible.

High affinity polyclonal and monoclonal anti-CTR antibodies developed here (Table 13.2) have been useful in animal studies with immunoblotting and IHC (Tikellis et al. 2003), particularly when the IHC signal was amplified using tyramide-based technology (Tolcos et al. 2003). More recently anti-human CTR monoclonal

Table 13.1 Tissues and cells found to express CTR

Tissue in adult	Cell type	Method of detection	References
Bone (human, mouse)	Osteoclasts	Tracer[a]	Gorn et al. (1995), Marx et al. (1972), Nicholson et al. (1986), Nicholson et al. (1987), Hattersley and Chalmers (1989), Zaidi et al. (1993), Quinn et al. (1999), Dacquin et al. (2004), Tobon-Arroyave et al. (2005), Vered et al. (2006), Granholm et al. (2008)
	Osteoclastoma, Giant cell tumours	IHC	
Kidney	Distal tubules	Tracer[a]	Lin et al. (1991), Marx et al. (1972), Sexton et al. (1987), Firsov et al. (1995), Wookey et al. (1996), Tikellis et al. (2003)
	Ascending loops of Henle	PCR	
	Collecting ducts	IHC	
	Cell line (LLC-PK1)		
Brain (mouse, rat, monkey, human)	Normal tissue	Tracer[a]	Fischer et al. (1981), Sexton et al. (1988), Nakamuta et al. (1990), Sexton et al. (1994), Sheward et al. (1994), Jagger et al. (1999), Nakamoto et al. (2000), Tolcos et al. (2003), Becskei et al. (2004), Paxinos et al. (2004), Spampinato et al. (1999)
	Human neuroblastoma cell line IMR 32	ISH	
		IHC	
Placenta (human, mouse)	Normal tissue	Tracer[a]	Nicholson et al. (1988), Kovacs et al. (2002)
		IHC	
Breast (human)	Normal tissue	Tracer[a]	Wang et al. (2004), Findlay et al. (1981), Findlay et al. (1980)
	Primary cancer	Laser capture/	
	Cell lines (T47D and MCF7)	PCR	
	BEN cells		
Ovary	Cell line (BIN-67)		Gorn et al. (1992)
Prostate (human)	Normal tissue		Wu et al. (1996), Thomas et al. (2007a), Thomas et al. (2007b)
	Primary cancer		
	Cell lines (PC-3M and LNCaP)		
Testis (human, mouse)	Normal tissue	Tracer[a]	Jagger et al. (1999), Chausmer et al. (1982), Nakhla et al. (1989)
	Leydig cells	Mouse transgenic	
Lung (porcine)		Tracer[a]	Fouchereau-Peron et al. (1981)

(continued)

Table 13.1 (continued)

Tissue in adult	Cell type	Method of detection	References
Bone marrow (human)	CD34+ blast cells	Northern blot	Brown et al. (1996), Marx et al. (1974), Moran et al. (1978), Body et al. (1990), Mould and Pondel (2003), Wookey et al. (2007), Silvestris et al. (2008)
	T-cells	PCR	
	Cell lines (K-562, RPMI 8866)	FACS	
	Cells of multiple myeloma	IHC	
Skeletal muscle (mouse)	Satellite cells	PCR IHC	Fukada et al. (2007)
Thymus (rodent)	Thymocytes/lymphoblasts		Whitfield et al. (1972)
Thyroid (human)	Cell lines (thyrotrophs)	Tracer[a]	Hanna et al. (1995), Frendo et al. (1994)
	Medullary thyroid carcinoma	PCR	

[a] Tracer used was [^{125}I]-salmon calcitonin, which binds tightly to CTR.

Table 13.2 List of antibodies developed to detect CTR for immunohistochemistry (IHC), immunofluorescence (IF) and flow cytometry (FACS)

	Targeted epitopes of CTR (refer to Fig. 13.1)				
	1	2	4	5	ELISA assays
Polyclonal antibody					
Anti-rat	PAb 189/10 (AbD Serotec, AHP 635 (Fukada et al. 2007))				PAb 189 (1: 640,000[a], rat epi-1 seq)
Anti-human	PAb 189/10 (Wookey et al. 2008)				(1:160,000[a], hum epi-1 seq)
Monoclonal antibodies					
Anti-human	MAb 31-01 (IgG2A) (AbD Serotec, MCA 2191 (Silvestris et al. 2008; Wookey et al. 2008))		MAb 1C11 (IgM) MAb 9B4 (IgG2A)	MAb 9H7 (IgG1)	MAb 31-01 1:120,000[a] MAb 1C11[b] MAb 9B4 1:20,000[a]
Anti-rabbit	MAb 31-01 (IgG2A)		MAb 1C11 (IgM) MAb 9B4 (IgG2A)		
Anti-rat	MAb 16-00 (IgG1) (AbD Serotec, MCA 2122 (Tikellis et al. 2003))	MAb 21-00, (IgG2A) (AbD Serotec, MCA 2192 (Tikellis et al. 2003))			MAb 16-00 1:80,000[a] MAb 21-00 1: 2,500[a]

[a] Dilution resulting in 50% colour formation in ELISA.
[b] NYA, not yet available.

antibodies raised against peptides that are unique and equivalent to an intra-cellular epitope (Epitope 1, Fig. 13.1) have been described for use with IHC (Wookey et al. 2008) and FACS analyses of permeabilized cells (Silvestris et al. 2008).

In the most recent development further monoclonal anti-human CTR antibodies, raised with conjugated peptides against epitopes within the extra-cellular domain, have proved useful for the FACS analyses of CTR-positive precursor blast cells (non-permeabilized) from human bone marrow (see Fig. 13.11).

13.3.3 Immunohistochemistry and Immuno-Fluorescence with High Affinity Anti-CTR Antibodies

Studies in the developing (Tolcos et al. 2003) and adult rat brain (Becskei et al. 2004) using these antibodies for IHC and signal amplification, on the one hand, and immuno-fluorescence on the other, respectively, have demonstrated high signal to background ratios. A further example of the definition of CTR+ve neural networks and individual neurons in the adult rat hindbrain is shown in Fig. 13.2.

A role for CTR+ve neurons and neural networks that extend from the forebrain, midbrain and hindbrain, and interface with components of the limbic system throughout the CNS (eg accumbens nucleus, substantia nigra) and hypothalamus has not been defined. A component of the network that extends from the *area postrema* (Riediger et al. 2001), with connections to the *parabrachial nucleus* and the hypothalamus (*arcuate nucleus*), is important for the transmission of signals from the activity of amylin in serum (co-secreted with insulin by the pancreatic islet cells) that influences feeding behaviour (Chance et al. 1991; Lutz et al. 1994, 1995).

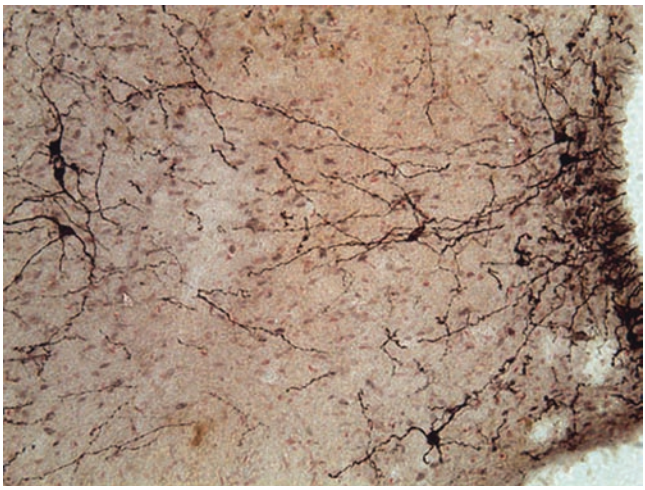


Fig. 13.2 CTR-immunoreactivity in the adult rat hindbrain, showing networks of CTR-positive neurons. See also Becskei et al. (2004)

13.3.4 Verification of Antibodies IHC Versus ISH, WB and FACS

There is a close distributional correlation between CTR expression at the mRNA and protein levels in the developing rat CNS (Tolcos et al. 2003). This finding strongly supports the specificity of the anti-human/rodent CTR polyclonal antibody (epitope 1, Table 13.2). This antibody has also been reported for use in Western blot of rat kidney proteins and detected a dominant band that had a mobility equivalent to 63 kD (Tikellis et al. 2003). A similar band was also detected from rabbit kidney and the cell line K562 (discussed below) using the monoclonal antibody MAb 31-01 (epitope 1, unpublished data).

More recently antibodies have been successfully raised against epitopes located in the extra-cellular domain of human CTR (epitope 4, MAbs 1C11 and 9B4, and epitope 5, MAb 9H7). In particular MAbs 1C11 (Fig. 13.11) and 9B4 have been tested in flow cytometry such as FACS analyses.

The myelogenous cell line K562 expresses CTR mRNA (Mould and Pondel 2003) and, when permeabilised, MAb 31-01 was successfully demonstrated in FACS analyses (Silvestris et al. 2008). We have recent data that confirm the efficacy of MAbs 9B4 and 31-01 with K562 cells in FACS analyses (unpublished data). Finally, both MAbs 1C11 and 9B4 have been used to characterise populations of CD34+ and CD34− cells from bone marrows of acute myeloblastic (AML) and lymphoblastic (ALL) patients with leukaemia (discussed below in relation to Fig. 13.11).

13.4 CTR Expression During Embryonic and Foetal Development

13.4.1 CT/CTR in Development of the Early Blastocyst and Gastrula

CTR mRNA is expressed by the blastocyst between the 1 and 8-cell stages (Wang et al. 1998). It is thought that this expression may lead to changes in the calcium activity that are important for processes in early embryonic life.

Calcitonin also influences the early intra-foetal development of Xenopus. The calcitonin-induced distortion of the head and abnormal development in Xenopus embryos is thought to result from inhibition of cell migration (including neural crest cells) into the head region during gastrulation (Burgess 1985).

13.4.2 CTR-β Gal Transgenic Mouse Models

The general pattern of expression of CTR during the second half of gestation in foetal mouse development was identified with the construction of CTR promoter/reporter gene chimeras in transgenic models. These results were generated from

two models in which the reporter gene β-galactosidase was regulated by either the porcine (Jagger et al. 1999) or human (Jagger et al. 2000) promoters of CTR. From these studies it was reported that CTR was expressed at foetal day 15.5 (E15.5) in limb buds, cornea, retina, skin, intercostal muscles, muscles of the face and limbs, dorsal root ganglia and extensively throughout the CNS (Jagger et al. 1999, 2000). These findings implied a potentially important role that CTR is likely to play in foetal morphogenesis and vitality.

Such a possible role was further emphasized by the finding that in the CTR−/− homozygote mouse, death occurred in utero (Dacquin et al. 2004).

13.4.3 CTR-Positive Precursor Cells That Migrate During Foetal Development

Cellular migration during foetal development is a fundamental phenomenon of organogenesis. There is little known about the factors that drive these migrations although the proliferation of the blast cell populations is considered an important factor (Heuckeroth and Pachnis 2006). A well-described example is the migration and role of neural crest cells in the development of many enteric tissues. Largely unknown factors are thought to influence the exact timing and pausing of blast cell migration, as well as proliferation and maturation. It could be expected that the epigenetic mechanisms that promote the final step(s) of maturation reside in the target tissue. Overall these spatio-temporal events are regarded as the key in the faithful reproduction of the foetal and adult body plans.

In parallel with immunohistochemical studies that investigated the development of CTR-positive neural networks in foetal CNS (Tolcos et al. 2003), three potential precursor cell populations that express CTR have been identified. These included populations of neuronal precursor cells (CNS, gut, eye), myelo-lymphoid precursors (liver and gut) and myoblasts.

CTR-positive neuroblasts (committed neural precursors) were identified early in the development of the CNS at E12/13 in the anlagen of the hypothalamus and pons (Tolcos et al. 2003). Further evidence of CTR-positive neuronal precursor cells was found with the identification of CTR-positive cells in the region of zones of proliferation (E19) at several locations adjacent to the ventricles (Tolcos et al. 2003). The late migration of these CTR-positve precursor cells at E19 is consistent with their commitment to the astroglial lineage although this possibility has yet to be verified.

During embryonic development in vertebrates, hematopoiesis occurs in two successive waves known as the primitive and the definitive haematopoiesis. In mammals, including mouse and human, primitive haematopoiesis occurs in the extra-embryonic yolk sac (Yoder et al. 1997; Samokhvalov et al. 2007; Yao et al. 2007; Zambidis et al. 2007). It is transitory and is mainly erythroid in lineage. Definitive haematopoiesis is initiated in the aorta-gonado-mesonephros (AGM) and it migrates subsequently to the foetal liver and the bone marrow.

This comprises the entire repertoire of the haematopoietic system throughout the lifespan of the organisms. Foetal haematopoietic stem cells are located transiently in foetal liver (about E17) and lymphoid precursors are derived from these myelo-lymphoid precursors (Nanno et al. 1994; Mebius et al. 2001; Mebius 2003).

Three cell types, which express CTR, were tentatively identified by morphology and location in E17 foetal liver, and these included megakaryocytes (Tober et al. 2007), Kupffer cells and putative myelo-lymphoid precursors (Fig. 13.3a). In adult, equivalent cell types (e.g. Kupffer cells, (Gale et al. 1978)) form part of the same lineage tree (Fig. 13.6). We have also found that megakaryocytes in adult mouse bone marrow are CTR-positive (unpublished).

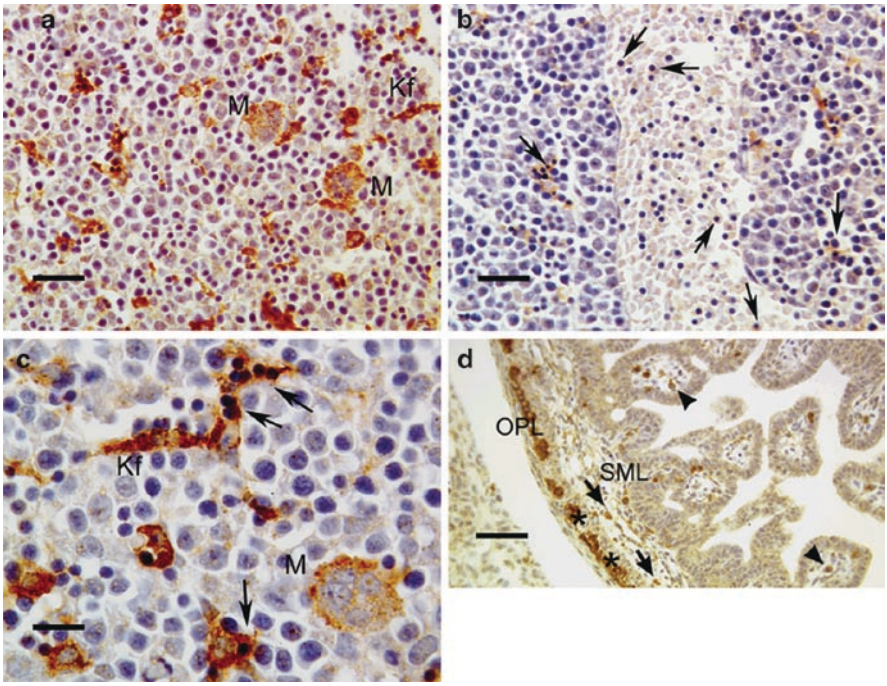


Fig. 13.3 Putative precursor cells in the embryo at day 17 and 19 (E17 and E19) of rat foetus identified using immunohistochemistry (DAKO CSA I amplification (Tolcos et al. 2003)) and the polyclonal antibody anti-CTR antibody (AHP 635, AbD Serotec, UK or 189/10, Welcome Receptor Antibodies, Australia). (**a**) CTR+ve cells in E17 rat liver: megakaryocytes (M) and Kupffer cells (Kf). Bar = 200 μm. (**b**) CTR+ve putative myelo-lymphoid precursors (*arrows*) in E17 rat liver, shown to be present both in the parenchyma and with reduced colour intensity in blood. Bar = 200 μm. (**c**) Higher magnification compared to (a) and (b), showing putative myelo-lymphoid precursors (*arrows*), megakaryocytes (M) and Kupffer cells (Kf). Bar = 100 μm. (**d**) E19 Gut CTR+ve neuroblasts (*arrows*) and lymphoid precursors (*arrowheads*) in the *lamina propria*. OPL = outer plexiform and SML = sub-mucosal layers. Immunoreactivity is also apparent in enteric ganglia (*asterisks*). Bar = 400 μm

13.4.4 Co-expression of CTR with the Proto-Oncogene RET in the Gut

The proto-oncogene RET is a membrane receptor that is activated as the result of the binding of the ligands glial cell-derived neurotrophic factor (GDNF), neurturin, artemin or persephin to the G-protein coupled receptors (GDNF family α receptors, GFRα-1 to 4, respectively).

The origins of the putative precursor of lymphocytes located in the lamina propria of gut (Fig. 13.3d (Adachi et al. 1997)) are thought to be derived from foetal haematopoietic precursors that have been identified in foetal liver (Nanno et al. 1994; Mebius et al. 2001; Mebius 2003). A subset of these precursor cells also express the proto-oncogene RET (Veiga-Fernandes et al. 2007). These ligands and their receptors are also found expressed by adult human immune cells (Vargas-Leal et al. 2005).

The model developed here includes the possibility that some of the lymphoid precursors found in the gut, which appear to be CTR-positive (Fig. 13.3d), are derived from the stem cells that occupy a niche in the rat foetal liver at E17.

It is of interest that neuroblasts in the developing foetal gut also express CTR (Fig. 13.3d) and are derived from neural crest cells. Post migration, precursor neural crest cells that eventually differentiate into glial and neuronal cells to form the enteric neural networks (Young et al. 1998; Young and Newgreen 2001), also express the proto-oncogene RET (discussed further below). Inactivating mutations of RET (Hirschsprung's disease (Takahashi et al. 1991)) and the RET−/− mouse result in an aganglionic syndrome.

13.4.5 CTR-Positive Structures in the Developing Thyroid

During the development of the thyroid, rosettes of follicular cells are found surrounding a central CTR-positive structure (Fig. 13.4). This interaction is believed to represent an important phase in the organogenesis of the thyroid. These observations provide an example of a further role of CTR in foetal development, namely in the organization of lobules of follicular cells around a central CTR-positive enteric neuron. A parallel involvement of neural crest derived cells in the induction of β-cell mass in the pancreas has been recently described (Nekrep et al. 2008). We have evidence of a similar spatial disposition of CTR-positive neurons that surround the β-cell mass in the E19 rat foetal pancreas (data not shown).

It is worth noting that CTR expression in the foetal peripheral nervous system (PNS) may be largely down-regulated with maturation of the organism. In that case, many components of the foetal PNS, including the enteric nervous system, could

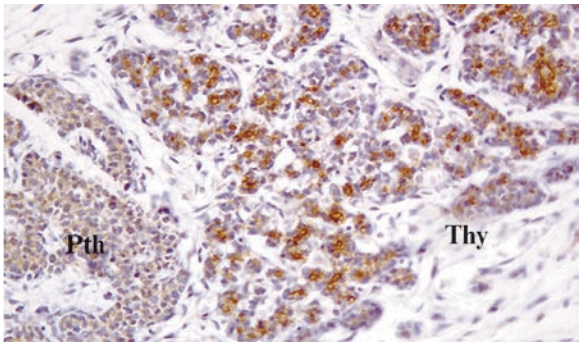


Fig. 13.4 Immunohistochemistry and PAb 189/10 (epitope 1) staining of the foetal rat thyroid (Thy) and parathyroid (Pth) at embryo day 19 (E19)

be regarded as transition structures that play a role in the development of the foetal body plan.

13.4.6 CTR Expression in the Developing and Adult Skeletal Muscle

At E12/13 CTR expression was found in the somitic myotome either using the CTR-ß galactosidase reporter construct in the transgenic mouse study (Jagger et al. 2000) or with IHC (data not shown) and the PAb 189/10 (Table 13.2). These precursor myoblasts migrate away to form skeletal muscle.

In Fig. 13.5 below similar staining is clearly identifiable in the nascent skeletal muscle at E19, as it is formed next to sites of attachment.

Interestingly, in adult skeletal muscle quiescent satellite cells in contrast to activated ones, express CTR (Fukada et al. 2007). These cells are important in muscle regeneration following injury.

13.4.7 CTR Expression in Postnatal Rodent Developing Kidney (Transient Up-Regulation)

In many growing and developing tissues in which CTR is expressed by expanding cell populations, CTR expression is highly elevated during growth and organogenesis. An example has been documented in our laboratory and was measured using quantitative PCR and image analysis following IHC. CTR mRNA was elevated fivefold during the postnatal period of renal development in the rodent, a period of rapid tubulogenesis and expansion (Tikellis et al. 2003).

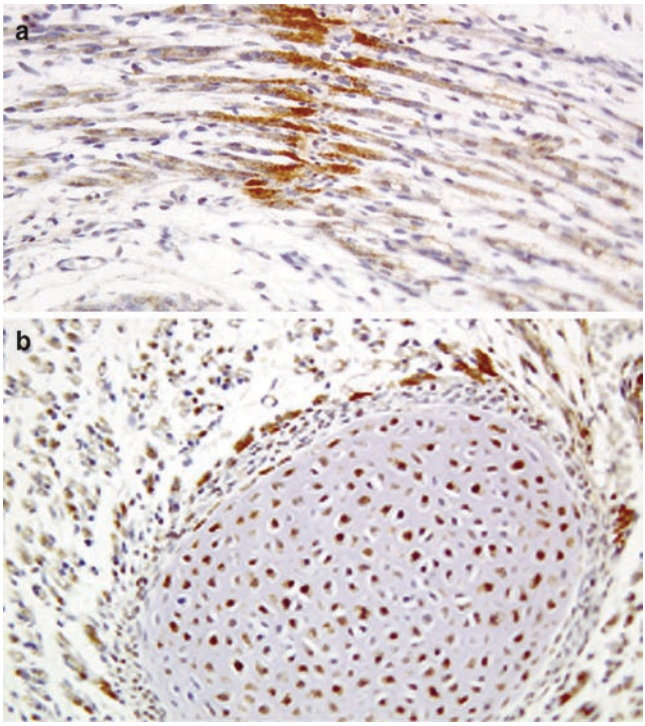


Fig. 13.5 Two examples of the expression of CTR in skeletal muscle at foetal day E19 that is increased at the sites of attachment

13.5 The Expression of CTR by Precursors and Progeny of the Haematopoietic Lineage

In Fig. 13.6 is shown a representation of the haematopoietic lineage tree that has been deduced for bone marrow stem cells and differentiated progeny. Committed haematopoietic progenitor cells have the potential for limited proliferation as well as commitment to differentiate into specific progeny. Many of these intermediates express the CD34 surface molecule, a marker expressed by most haematopoietic progenitor cell populations. CD 34 is a glycoprotein that has been classified as an adhesion molecule, which functions in the attachment of these cells to the endothelial surface of blood vessels where they may differentiate into endothelial cells or foam cells (Daub et al. 2006). We have preliminary evidence that CTR can be expressed by subpopulations of haematopoietic progenitor cells and this may determine the destination or micro-environment into which the cells migrate, and subsequently participate in physiological and pathophysiological functions.

For instance macrophages, Kupffer cells (Gale et al. 1978) that line the hepatic sinusoids, foam cells (Daub et al. 2006) in cardiovascular disease (CVD, discussed further below) and osteoclasts (Sorensen et al. 2007) all are derived from monocytes.

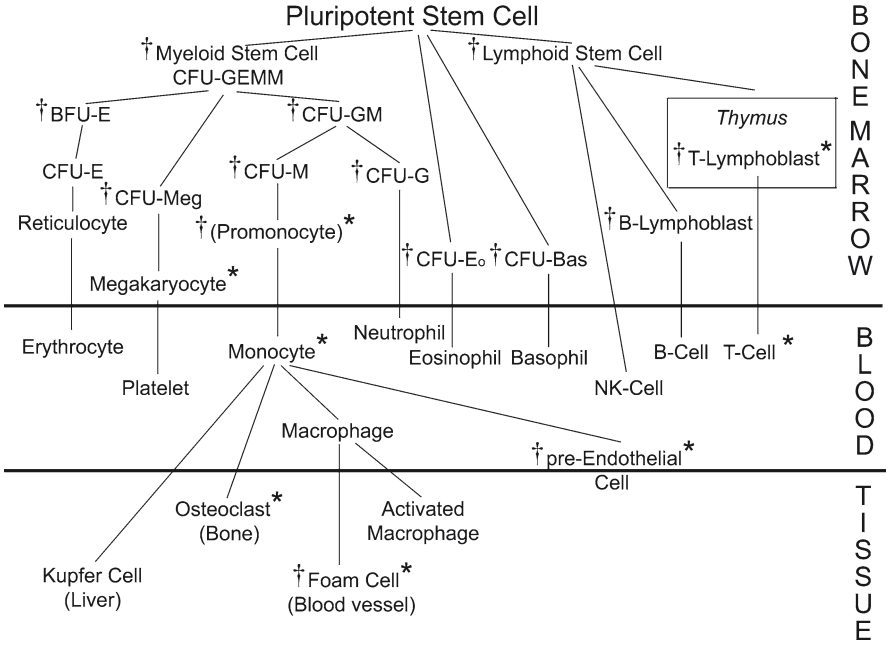


Fig. 13.6 Schema of the haematopoietic lineage. Cell types that express (*) CTR and (†) CD34 are indicated (Wookey, 2009)

In the foetal liver (Fig. 13.3) it appears that Kupffer cells exhibit transient expression whereas megakaryocytes may undergo prolonged expression of CTR as a large proportion of megakaryocytes in adult bone marrow of mice are also CTR-positive (data not shown, see also Fig. 13.10a-c).

13.5.1 T Lymphocytes

CT induces proliferation of T lymphoblasts (Whitfield et al. 1972) in rat thymus and presumably these cells express CTR. Normal human T lymphocytes express high affinity CT binding sites (Body et al. 1990), a characteristic of CTR. In tonsillar tissue, increasing CT is found to be expressed by endothelial cells with inflammation and coincides with the migration of lymphocytes across high endothelial venules (Ozbilgin et al. 2006).

13.5.2 CTR expression and haematopoietic lineages

These data suggest that CTR may be expressed in two phases in relation to lineage restriction. The first corresponds to progenitor cell stages within bone marrow that may spill over into blood in disease states. As progeny mature and become inte-

grated into target tissues there may be a second phase that is determined largely by the local tissue micro-environment. Such biphasic expression has previously been noted in the developing kidney at a time of rapid tubulogenesis (Tikellis et al. 2003). The finding of CTR expression by malignant plasma cells, capable of osteolysis, that were obtained from patients with multiple myeloma (Silvestris et al. 2008), may represent a version of the second phase but expression in the case of this disease may be independent of the tissue micro-environment.

The potential to express CTR by some of the terminal differentiated progeny as shown in the lineage tree (Fig. 13.6) is yet to be determined, such as expression by eosinophils, neutrophils and basophils although we have anecdotal evidence that the former two do express CTR.

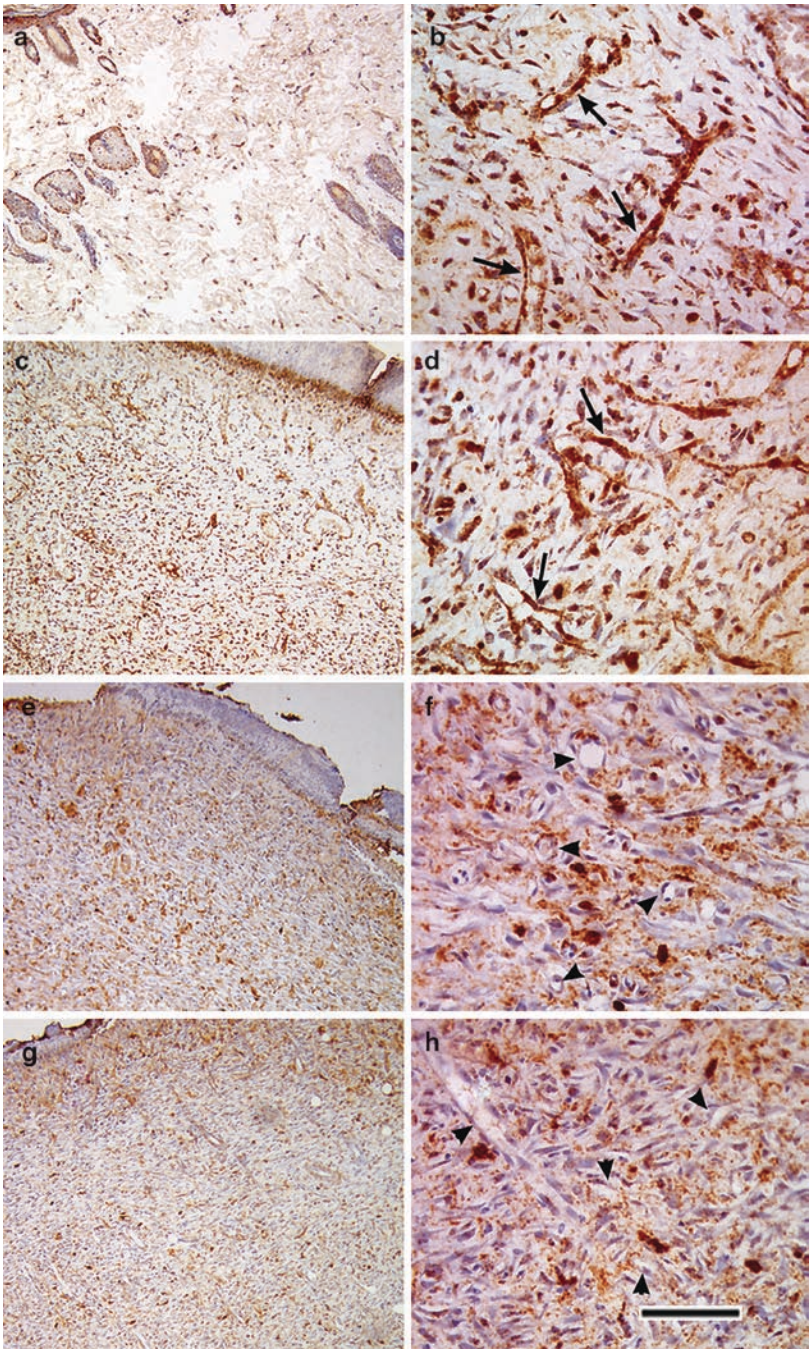
In summary, several types of differentiated haematopoietic cells and their progenitors have been shown to express CTR in various adult tissues. Thus, the potential expression of CTR is likely to be a basic characteristic associated with haematopoiesis.

13.6 Wound Healing, a Mouse Model

A subpopulation of circulating CD34-positive cells represents functional endothelial precursors that express VEGFR-2 (Peichev et al. 2000). CT stimulates angiogenesis with HMEC-1 cells that also express CTR (Chigurupati et al. 2005). In Fig. 13.7b and d endothelial cells that line nascent blood vessels and fibroblasts of this granulation tissue of healing wound (day 7) express CTR compared to normal skin (Fig. 13.7a). This expression is completely down-regulated by day 12 when healing is almost complete (Fig. 13.7g and h).

It is worth noting that circulating fibrocytes are recruited into skin lesions where they contribute with local fibroblasts (from surrounding tissue) in the healing process (Peters et al. 2005; Mori et al. 2005). These are likely to be descended from mesenchymal stem cell populations that are unrelated to the haematopoietic lineage (Fig. 13.6). In particular, within the healing granulation tissue CTR-positive cells have been identified that resemble fibroblasts in terms of morphology.

Fig. 13.7 The healing skin wounds of mice are represented in these images. Control tissue from a similar subcutaneous region of the back is shown in panel **a**. Shown in panels **b** and **d** are images from the same field as panel **c** seven days after healing had begun. Arrowed are examples of CTR-positive cells lining nascent blood vessels. Also apparent within this granulation tissue are CTR-positive cells that appear by shape as elongated myofibroblasts. By day 10 (panels **e** and **f**) the intensity of CTR expression had been reduced in the region of healing particularly in the endothelial cell population (*arrowheads*). Shown in panels **g** and **h** is the region of a wound 12 days after healing had commenced and there is evidence of a further decrease in the intensity of staining and/or the number of CTR-positive cells. Scale bar in **a**, **c**, **e** and **g** = 190 μm and **b**, **d**, **f** and **h** = 50 μm


a
b
c
d
e
f
g
h

This period of healing is characterised by a rapid expansion in the populations of CTR-positive cells.

13.7 Cardiovascular Disease (CVD)

Circulating BMSCs contribute to the endothelium of atherosclerotic plaque (Xu et al. 2003) and neointima (Campbell et al. 2000, 2001).

13.7.1 Rabbit Model of Atherosclerosis

The rabbit model of early CVD pathogenesis has many features similar to humans including the presence of reverse cholesterol transport. Of the precursor cells invading nascent atherosclerotic plaque, several cell types are CTR-positive (Fig. 13.8a). These include endothelial cells and foam cells that are derived from blood borne monocytes (CD34-positive, (Daub et al. 2006)), and fibroblasts that probably have originated from mesenchymal stem cells.

13.7.2 More Advanced Human CVD: CTR Expression in the Media and Adventitia, and Calcification

The severity and extent of calcification of vessels reflect the atherosclerotic burden, and strongly and independently predict cardiovascular morbidity and mortality (Sangiorgi et al. 1998). Vascular calcification is now recognized as a pathobiological process sharing many features with embryonic bone formation, in which endothelial, mesenchymal and haematopoietic cells interact in response to mechanical, inflammatory, metabolic and morphogenic signals in the arterial wall and govern mineralisation (Demer and Tintut 2008).

In advanced examples of human CVD, CTR-positive, nucleated cells in the blood have been found associated with the endothelium of a human radial (Fig. 13.9a and b) and internal mammary arteries (Wookey et al. 2008). Similar CTR-positive nucleated cells have also been noted attached to the endothelium lining the lumen of human diseased radial arteries (Fig. 13.9b). The expression of CTR in putative tubules that are found in the diseased media (Fig. 13.9a) may be a pre-condition or intermediate step in processes that lead to calcification of the vessel walls (Wookey et al. 2008).

In conclusion, nucleated progenitor cells and/or (pro) monocytes (CTR+/CD34+), observed to be attached to the luminal endothelium, are recruited into regions of CVD and may differentiate into endothelial cells and/or foam cells (Daub et al.

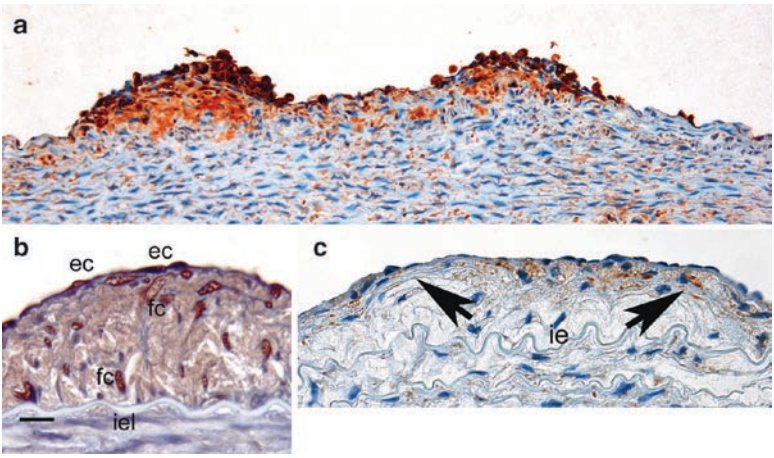


Fig. 13.8 Diseased rabbit aorta with atherosclerotic plaque (Zulli et al. 2004, 2005; Wookey et al. 2009). (**a**) Nascent atherosclerotic plaque showing CTR-immuno-reactivity (CTR-ir, MAb 1C11, epitope 4) associated only with cells on the surface of plaque but absent from adjacent endothelium. (**b**) CD 34+/CTR+ endothelial cells (ec) and CTR+ foam cells (fc) within more advanced plaque (CTR-ir, MAb 31-01, epitope 1). Bar in B = 100μm. (**c**) CTR-ir is down regulated in endothelium and remnants of foam cells in plaque stabilised with smooth muscle cells (*arrows*), i.e. internal elastic layer

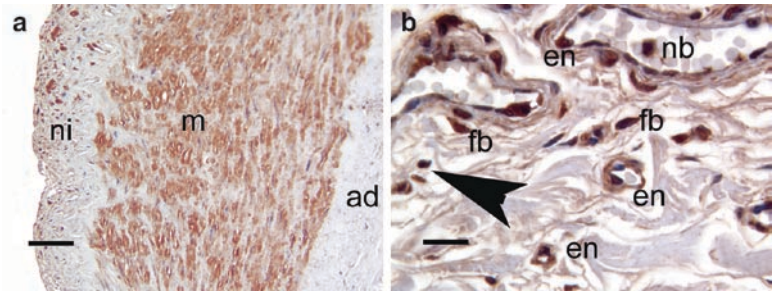


Fig. 13.9 (**a**) Human diseased radial artery. CTR-positive cells in the neo-intima (ni), and structures within the media (m) that contain CTR+ve smooth muscle cells, ad = adventitia (Wookey et al. 2008). Bar = 250 μm. (**b**) At higher magnification, the adventitia surrounding the media as shown in A, contains CTR-positive endothelium (en), CTR-positive fibroblasts (fb), an example of a blood-borne CTR-positive cell (nb) and smaller CTR+ cells within the parenchyma (arrowhead). Bar = 75 μm (published with the kind permission of Wiley-Blackwell Publishing (Wookey et al. 2008))

2006). These cells and/or differentiated progeny play a role in the early stages of disease in the arterial walls, whereas CTR-positive cells and structures within the media are also involved later in more advanced CVD (Wookey et al. 2008).

13.8 Tumourogenesis

The role of cancer stem cells remains controversial and migration of other cells are cellular events that may contribute to the formation of solid tumours (Fomchenko and Holland 2005; Kaplan et al. 2005). These are therefore complex tissues that are comprised of primary tumour cells as well as primitive cells and other cells recruited into the tumour. These events may share some features (albeit with apparent disorganized morphology) with the paradigms and processes of foetal organogenesis.

Primary tumour cells from breast (Wang et al. 2004; Gillespie et al. 1997) and prostate (Thomas et al. 2006) cancers, and interestingly, malignant plasma cells from multiple myeloma (Silvestris et al. 2008) express CTR. In the case of prostate, expression of CTR is thought to correlate with the malignant potential of the tumour whereas in the study with multiple myeloma, CTR expression was related to osteolytic activity of the plasma cells and clinical outcomes.

Many cell lines that are derived from different tumours and can be cultured in vitro, express CTR. A short list of those that have CT binding sites or express CTR includes osteoclastoma (Nicholson et al. 1987) and central giant cell granuloma cells (Gorn et al. 1995; Vered et al. 2006), breast cancer-related cell lines MCF-7 (Chen et al. 1997) and T47D (Kuestner et al. 1994), an ovarian carcinoma cell line (Gorn et al. 1992) and thyrotrophs (Hanna et al. 1995). Leukaemic cell lines express CT binding sites (Marx et al. 1974; Moran et al. 1978), CTR mRNA (Mould and Pondel 2003; Silvestris et al. 2008) and CTR protein detected by FACS analysis (Silvestris et al. 2008). However, it is not clear what role the primary cells that gave rise to these cell lines, might have played in tumourogenesis.

13.8.1 CTR Expression in Leukaemia

Initially, evidence of CTR expression in blast cells from patients with AML was found with IHC staining in a limited number (five out of eight total) of bone marrow aspirates (Wookey et al. 2007) (Fig. 13.10). In BM aspirates of similar patients, significant populations of CD34−/CTR+ cells have also been discovered.

Subsequently, using FACS analyses (examples below in Fig. 13.11) of a larger number of BM samples (from ALL and AML patients), evidence of CTR+/CD34+ and CTR+/CD34− populations, was found. These data are summarized in Table 13.3.

These findings are of interest because they demonstrate that CTR is expressed by some primary tumour blast cells whose existence in the corresponding tissue may be the primary cause of the disease, and in the case of multiple myeloma, contributes to clinical manifestations such as osteolysis (Silvestris et al. 2008). In ALL and AML, CTR may be expressed by cells of the BMSC lineage (Fig. 13.6) as an incidental marker rather than being oncogenic. The role of CTR in these blast cells is yet to be determined.

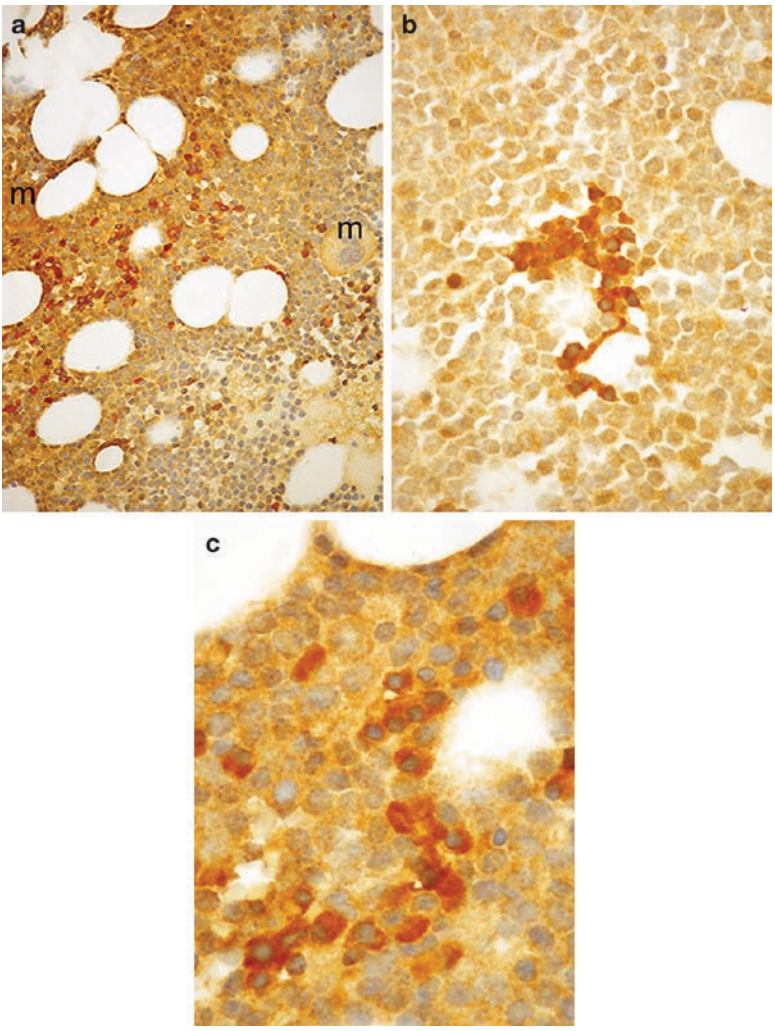


Fig. 13.10 Bone marrows from 3/8 AMLs (5/8 were identified as CTR-positive). (**a**) = Mag X10, m = megakaryocyte; (**b**) = Mag X20; (**c**) = Mag X40

13.8.2 The Expression of the Proto-Oncogene RET in Normal BM and Leukaemia

In the BM environment, where haematopoiesis is normally tightly regulated, there is thought to be an interaction between stromal cells and RET-positive haematopoietic blast cells, which plays a role in the regulation of the differentiation of myeloid precursors and T-cells (Gattei et al. 1997; Nakayama et al. 1999). RET expression

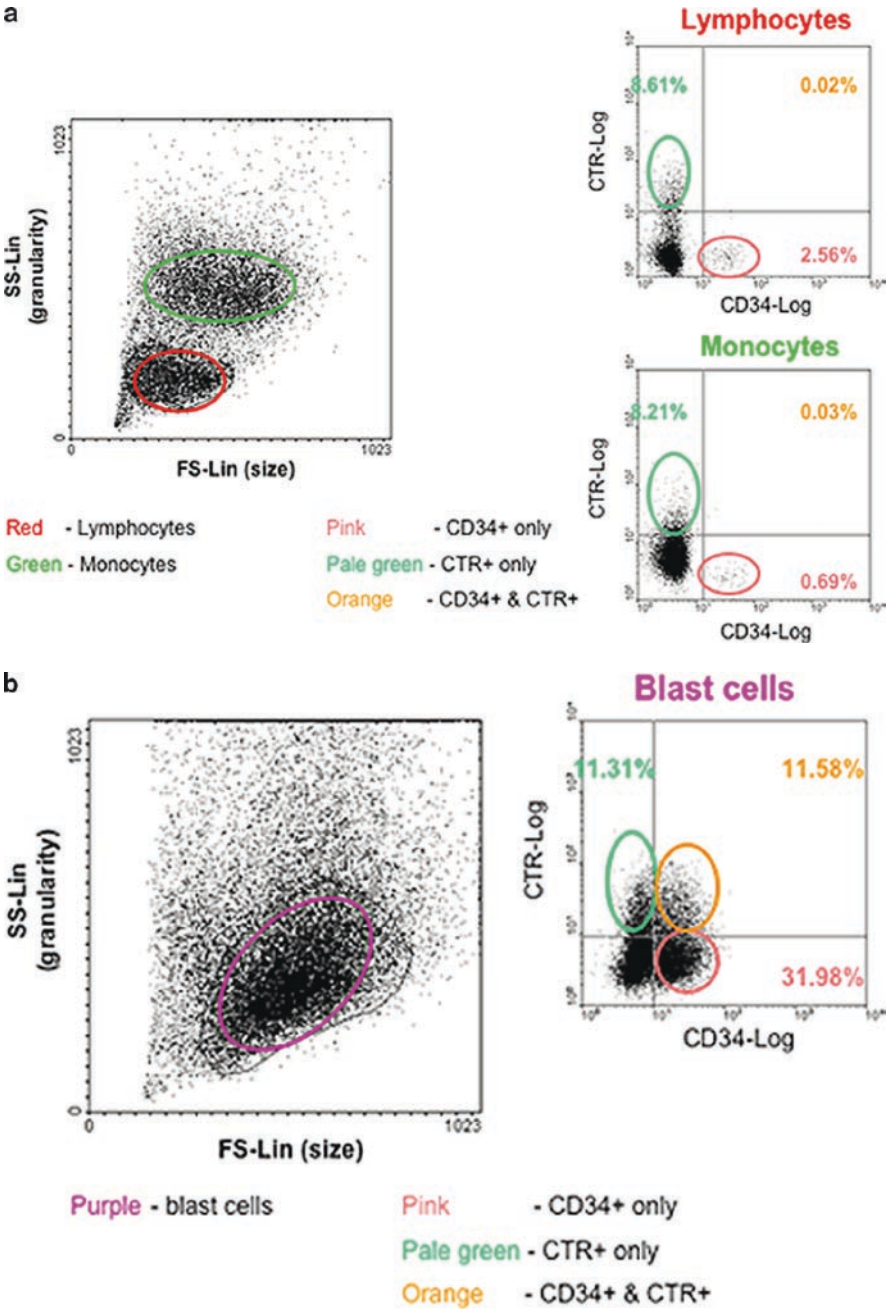


Fig. 13.11 FACS analysis using anti-human CTR antibody (MAb 1C11) and anti-CD34 antibody. (**a**) normal PBSCs, lymphocytes (CTR+/CD34+, 0.02%; CTR+/CD34-, 8.6%) and monocytes (CTR+/CD34+, 0.03%; CTR+/CD34–, 8.2%); (**b**) AML bone marrow (CTR+/CD34+, 11.3%; CTR+/CD34–, 11.6%); (**c**) ALL bone marrow, population 1 (CTR+/CD34+, 6.1%; CTR+/CD34–, 0.5%), population 2 (CTR+/CD34+, 10.4%; CTR+/CD34–, 0.4%)

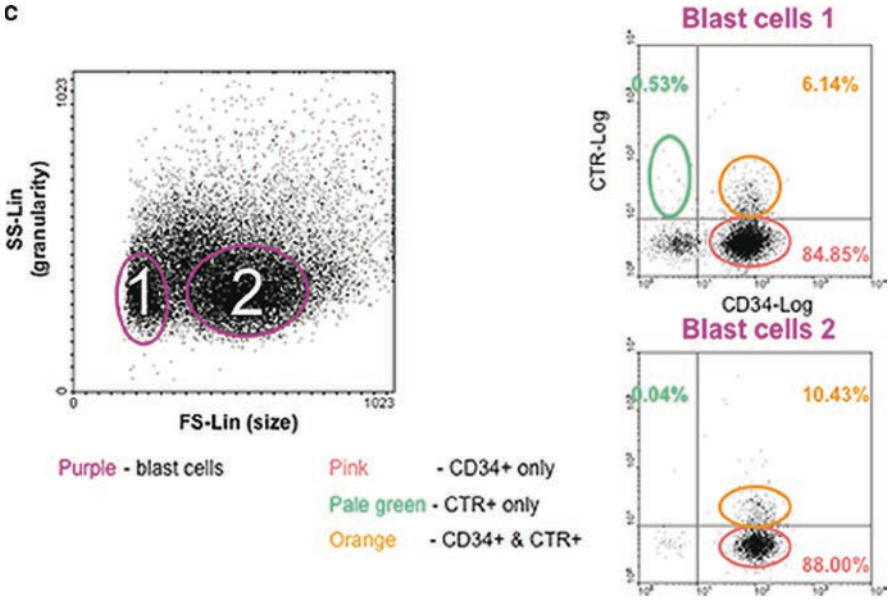


Fig. 13.11 (continued)

Table 13.3 Summary of human leukaemic and normal bone marrow samples analysed by FACS to determine the sizes of the CTR+ blast cell (CD34+) populations

Type of leukemia	Sub-category	Number of samples	% CTR+/CD34+[a]		% of disease category	
			0.5–2.5%	>2.5–15%	0.5–2.5%	>2.5%
ALL	Not available	12	4	5	33%	42%
AML	–	8	1	1		
	MLD	2	1	1		
	M1	1	1		31%	12.5%
	M2	2	1	0		
	M5	3	1	0		
Normal BM		6	2	0	33%	0%

Data accumulated over the period September 2006 to May 2007, University of Hong Kong.
[a]This is the range of CTR+/CD34+ cells given as a percentage of the gated population in PI–negative cells.

was also detected in B-cells and monocytes (Vargas-Leal et al. 2005). Important for this discussion, RET is expressed by leukaemic blast cells but confined to the myeloid lineage in AML. Given these properties of RET expression it is somewhat surprising that mutations in the RET gene have not (yet) been associated with any form of leukaemia (Visser et al. 1997).

In summary, it appears that RET expression is confined to a limited number (subset) of cell intermediates and progeny of the haematopoietic lineage tree in which there is more extensive expression of CTR.

13.9 Possible Cellular Mechanisms Involving CTR

13.9.1 Retardation of the Cell Cycle

A calcitonin response element (Sp1 binding site) was identified in the promoter of the human $p21^{WAF1/CIP1}$ gene encoding a cyclin-dependent kinase inhibitor (Evdokiou et al. 2000). Calcitonin induced cell cycle arrest at the G_2/M phase in cells transfected to express the insert-negative isoform of CTR. Such a mechanism may be relevant to CTR-positive precursor cells, including neuroblasts, precursors of the haematopoietic lineages and quiescent satellite stem cells associated with muscle (Fukada et al. 2007) (discussed above). Such a mechanism and the reduction in survival (caspase-3 independent) (Findlay et al. 2002) may be important control mechanisms in haematopoiesis and CTR may be down-regulated or inactivated during leukaemogenesis.

It is yet to be established whether such mechanisms also play a role in the control of cellular proliferation during wound healing and tubulogenesis in the developing kidney.

13.9.2 Migration and Recruitment of Precursor Cells and/or Progeny

There are several instances and reports that provide evidence for the involvement of CTR in mechanisms of cell migration. For instance, immature monocytes mobilised in blood are CD34+/CTR+ and attach to the endothelial layer of diseased blood vessels (CVD, Figs. 13.8 and 13.9 (Wookey et al. 2008)). Second, CT promotes the invasiveness of prostate cancer cells that express CTR (Thomas et al. 2007a). Third, endothelial cells that line blood vessels of the tonsils express CT and promote the migration of lymphocytes (Ozbilgin et al. 2006).

13.9.3 Promotion of Differentiation, for Instance Progeny of the Haematopoietic Lineage

The expression of CTR is thought an important factor in the control of the terminal differentiation of osteoclasts (derived from monocytes) and osteolysis (Hattersley and Chalmers 1989; Zaidi et al. 1993; Dacquin et al. 2004; Granholm et al. 2007, 2008). For other progeny of the same haematopoietic lineage that express CTR, such as lymphocytes, megakaryocytes, foam and endothelial cells, CTR may also play a role in the terminal differentiation process.

13.10 The Micro-environment and CTR-Positive Cell Types

The induction of CTR expression may be dependent on cognate ligands and/or other factors within a defined micro-environment as well as the lineage of the recruited cells (Wookey, 2009). In the foetus, such niches are evident from the expression of CTR by hemangioblasts in liver and later in bone marrow. It remains to be tested whether hemangioblasts of the AGM region and earlier in the yolk sac, also express CTR.

In the adult, within the bone and the BM niches, it is well known that endogenous cells provide essential growth and survival factors such as stromal cell-derived factor (SDF-1, CXCL12), RANKL, GDNF and many other factors. These factors contribute to the tight regulation of the populations of cell types and haematopoiesis in general.

During the expansion of the CTR-positive BM populations in diseases such as leukaemia, CVD and in an inflammatory response including wound healing, spill-over of precursor cells into peripheral blood occurs. In instances when CTR functions in migration it is likely that the cognate high affinity ligand for CTR is synthesized within the target tissues (such as CVD (Wookey et al. 2008, 2009) and tonsils (Ozbilgin et al. 2006)) that recruit CTR-positive cells.

It is proposed that within the micro-environment of atherosclerotic plaque such ligands will be expressed, and that CTR contributes to a homing mechanism for precursor cells recruited into these tissues. These events may be important for our understanding of the principles of healing in diseased vessels and in the potential treatment of CVD that is a high risk factor for stroke.

13.10.1 What are the Implications for the Co-expression of CTR and RET?

There are several interacting systems that might influence the expression of CTR. For instance, CTR (CT binding sites) is down-regulated by CT and corticosteroids in osteoclasts (Wada et al. 1996, 1997, 2001). This could influence CTR expression in the uterus where progesterone stimulates CT expression (Ding et al. 1994).

Other factors may also influence expression including putative CT-like peptides that are thought to be expressed in some tissue for instance brain and diseased blood vessels as described above.

Interestingly, GDNF and persephin stimulate CT expression in thyroid cells (Akeno-Stuart et al. 2007) which may lead to local and/or distant down-regulation of CTR.

It is feasible that one of the ligands (GDNF, persephin, artemin, neurturin) that modifies RET activity in the foetal enteric nervous system and other tissues in which there is co-expression, also influences local synthesis of a CT-like ligand of CTR.

13.11 Conclusions and Future Perspectives

Evidence for the widespread expression of CTR has been reviewed and was the result of many studies over the last five decades (Table 13.1). CTR was found to be expressed by different cell types that performed several developmental functions as well as the endocrine functions as originally defined. The former can be grouped into several categories. In the foetus, specific subpopulations of neuroblasts of the CNS and the eye, neural crest cells that differentiate into the neuroblasts of the enteric nervous system, myelo-lymphoid precursors of the foetal liver and skeletal myoblasts, express CTR.

Expression of CTR coincides with the migration of precursor cells, important in the processes of organogenesis in the developing foetus. In the case of the neural crest cells that migrate into the gut and give rise enteric neurons, these express CTR. It is yet to be established whether this expression is transient, being down-regulated around birth. These structures may play a central role in the organization of some developing tissues for example the thyroid (Fig. 13.4). Thus expression of CTR may also constitute distinctive transitional structures in peripheral developing tissues.

The expression of the proto-oncogene RET appears to overlap that of CTR in a spatio-temporal sense in some branches of the haematopoietic lineage tree but they may function independently of each other. As noted above these two membrane proteins are co-expressed in other foetal cell populations such as neural precursors in the enteric nervous system that are derived from post-migratory neural crest cells. It remains to be determined what significance co-expression might have for organogenesis.

In the adult, CTR is expressed by a many different cell types including precursor cells and their differentiated progeny associated with the haematopoietic lineage. Expression of CTR may be separated into two phases. In some tissues undergoing normal physiological functions such as wound healing and in diseases as discussed above, the expression of CTR may be part of a mechanism involved in the recruitment and migration of blood-borne precursors important for subsequent healing or that play a significant role in the aetiology of disease. In CVD expression of CTR is found in the early phases of atherosclerosis during recruitment of precursors and again later in more advanced arterial disease prior to calcification of the vessels.

CTR is expressed by malignant cells from a significant proportion of patients with AML and ALL. It will be important to determine the functional significance of CTR expression in the etiology of acute leukaemic and whether CTR expression can be targeted for prognostic purposes, predictive outcomes for patients and perhaps a role in therapy.

It will be interesting to identify the putative high affinity ligands for CTR, which may be expressed in different target tissues and that promote the recruitment of CTR-positive precursor cells.

Finally, transient expression of CTR mRNA and/or protein may be an important mechanism for the control of physiological events such as renal development (Tikellis et al. 2003), wound healing and T-cell migration (Ozbilgin et al. 2006). On the other hand, progression to disease may be accompanied by up-regulated and prolonged expression of CTR by specific cell types in diseased tissues.

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