



## The transcription factors Snail1 and Snail2 repress vitamin D receptor during colon cancer progression<sup>☆</sup>

María Jesús Larriba<sup>a,\*</sup>, Félix Bonilla<sup>b</sup>, Alberto Muñoz<sup>a</sup>

<sup>a</sup> Instituto de Investigaciones Biomédicas "Alberto Sols", Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Arturo Duperier 4, 28029 Madrid, Spain

<sup>b</sup> Hospital Universitario Puerta de Hierro Majadahonda, Joaquín Rodrigo 2, 28222 Majadahonda, Spain

### ARTICLE INFO

#### Article history:

Received 19 October 2009

Accepted 31 January 2010

#### Keywords:

Snail1

Snail2

Vitamin D receptor

1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub>

Colon cancer

### ABSTRACT

Vitamin D receptor (VDR) mediates the antitumoral action of the active vitamin D metabolite 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>). However, VDR expression is lost during colon cancer progression, possibly causing unresponsiveness to 1,25(OH)<sub>2</sub>D<sub>3</sub>. Although several mechanisms responsible for resistance to 1,25(OH)<sub>2</sub>D<sub>3</sub> action in different types of cancer had been reported, none explained the loss of VDR expression. We have found that the transcription factors Snail1 and Snail2, known as inducers of epithelial-to-mesenchymal transition (EMT), inhibit VDR expression and block 1,25(OH)<sub>2</sub>D<sub>3</sub> action in colon cancer cells. Snail1 and Snail2 have an additive repressing effect on VDR gene promoter. These effects are specific to the Snail family, as other transcription factors that function as EMT inducers do not inhibit VDR expression in colon cancer cells. Moreover, we also found that the RNA expression of *SNAIL1* and *SNAIL2* is upregulated in human colorectal tumors and inversely correlates with that of VDR. Our results suggest that high levels of *SNAIL1* and *SNAIL2* are a probable cause of VDR downregulation and 1,25(OH)<sub>2</sub>D<sub>3</sub> unresponsiveness in colon cancer. In addition, they may contribute to the improvement of protocols for the clinical use of vitamin D compounds, as they indicate that advanced colon cancer patients overexpressing *SNAIL1* and *SNAIL2* are not suitable candidates for this therapy.

© 2010 Elsevier Ltd. All rights reserved.

### 1. Introduction

1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) and a number of less calcemic analogs are in clinical trials as anticancer agents against colon cancer and other neoplasias based on their antiproliferative, prodifferentiation, pro-apoptotic and antimetastatic activity in cultured cells and experimental animal models [1,2]. We have shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits proliferation and promotes differentiation of human colon cancer cells *via* the induction of several genes such the invasion suppressor *CDH1*/E-cadherin and the candidate tumor suppressor *CST5*/cystatin D, and by the antagonism of the Wnt/ $\beta$ -catenin pathway that is aberrantly activated in most colon tumors [3–5].

Although preclinical studies are promising, initial clinical trials in colon cancer have shown acceptable toxicity but low activity of vitamin D compounds [1]. As it is usual in the development of

new antitumoral drugs, patients enrolled in these trials had not responded to any other therapy and they were unselected in terms of putative responsiveness to 1,25(OH)<sub>2</sub>D<sub>3</sub>. A better understanding of the physiology of the vitamin D system and the identification of the mechanisms responsible for resistance to 1,25(OH)<sub>2</sub>D<sub>3</sub> may help us to design future clinical trials more rationally.

### 2. Vitamin D receptor is downregulated during colon cancer progression

Most, if not all, 1,25(OH)<sub>2</sub>D<sub>3</sub> effects are mediated by the vitamin D receptor (VDR), a transcription factor of the nuclear receptor superfamily. Thus, cellular VDR expression is required for a clinical response to vitamin D compounds. VDR is expressed in normal colon epithelial cells and also in some colon cancer cells. Remarkably, elevated VDR expression is associated with high differentiation, absence of node involvement and favourable prognosis in colorectal cancer [6,7]. However, VDR expression is downregulated during colon cancer progression [8–10] probably causing 1,25(OH)<sub>2</sub>D<sub>3</sub> unresponsiveness.

Although VDR downregulation in colon cancer was first described more than a decade ago, its molecular basis had remained elusive. Deletions, rearrangements or point mutations affecting the coding region of the VDR gene had not been found in cancer. Neither

Abbreviations: EMT, epithelial-to-mesenchymal transition; VDR, vitamin D receptor; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>.

<sup>☆</sup> Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

\* Corresponding author. Tel.: +34 91 5854452; fax: +34 91 5854401.

E-mail address: [mjlarriba@iib.uam.es](mailto:mjlarriba@iib.uam.es) (M.J. Larriba).

had epigenetic silencing nor transcriptional repression mechanisms been described. Several polymorphisms had been described in the *VDR* gene, some of which have been associated with increased risk of breast, prostate and colon cancer. However, their consequences for *VDR* expression or functionality, and therefore their implication in the development of  $1,25(\text{OH})_2\text{D}_3$  resistance remain to be established [11,12].

### 3. The transcription factors Snail1 and Snail2 repress *VDR* expression in colon cancer cells

A novel mechanism responsible for *VDR* downregulation in colon cancer has emerged in recent years. Our group has revealed that the transcription factors Snail1 and Snail2 (also known as Snail and Slug) encoded by *Snai1* and *Snai2* genes, respectively, bind to the promoter region of exon 1a of human *VDR* gene and repress its expression. The repressive effects of Snail1 and Snail2 on *VDR* gene promoter are quantitatively similar and are mediated by three E-boxes (CAGGTG/CACCTG, reported binding sites for Snail factors) present in the first 400 nucleotides of human *VDR* promoter. In addition, we found that both transcription factors cooperate to repress *VDR* promoter, showing an additive effect [13,14].

We also found that the overexpression (by means of retrovirus-mediated gene transfer) of Snail1 or Snail2 in human colon cancer cells decreases *VDR* RNA and protein expression and strongly inhibits the regulation of  $1,25(\text{OH})_2\text{D}_3$  target genes such as *CDH1*/E-cadherin,  $p21^{CIP1}$  and *CYP24A1*/ $1,25(\text{OH})_2\text{D}_3$ -24-hydroxylase. Accordingly, Snail transcription factors block the epithelial differentiation induced by  $1,25(\text{OH})_2\text{D}_3$  in colon cancer cells (Fig. 1) and also the inhibitory effect of  $1,25(\text{OH})_2\text{D}_3$  on Wnt/ $\beta$ -catenin signaling pathway [13–15].

### 4. Relevance of *VDR* downregulation for the epithelial-to-mesenchymal transition induced by Snail1 and Snail2

Snail1 and Snail2 belong to the Snail family of zinc-finger transcription factors and modulate processes that imply cell movement during embryonic development and tumor progression. Cellular overexpression of Snail1 or Snail2 induces the epithelial-to-mesenchymal transition (EMT), which entails the loss of epithelial characteristics and the acquisition of a mesenchymal fibroblastic phenotype [16,17]. Therefore, the expression of Snail1 or Snail2 in carcinoma cells promotes their migratory and invasive properties favouring tumor invasion and metastasis. Accordingly, the aberrant

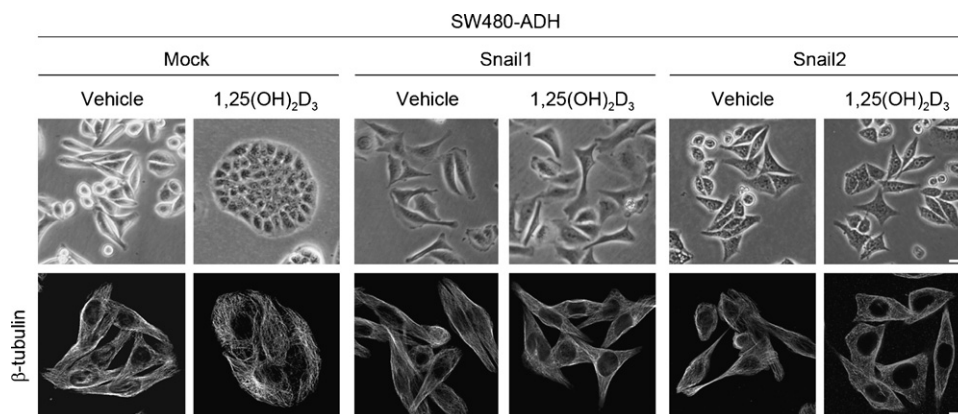
overexpression of SNAIL1 and/or SNAIL2 has been observed in different types of carcinomas and is frequently associated with invasiveness, metastasis and poor prognosis [16,17].

EMT induction is not an exclusive effect of Snail factors. Other transcriptional regulators such as E47, Twist1 and E2-2 (members of the basic-helix–loop–helix family), and Zeb1 and Zeb2 (of the Zeb family) also promote this process. EMT is mainly the result of transcriptional changes induced by these factors such as the repression of *CDH1*/E-cadherin, *OCLN*/occludin, several claudins and other epithelial genes and the induction of mesenchymal genes such as *FN1*/fibronectin, *VIM*/vimentin, *LEF1* and numerous matrix metalloproteases [16,17].

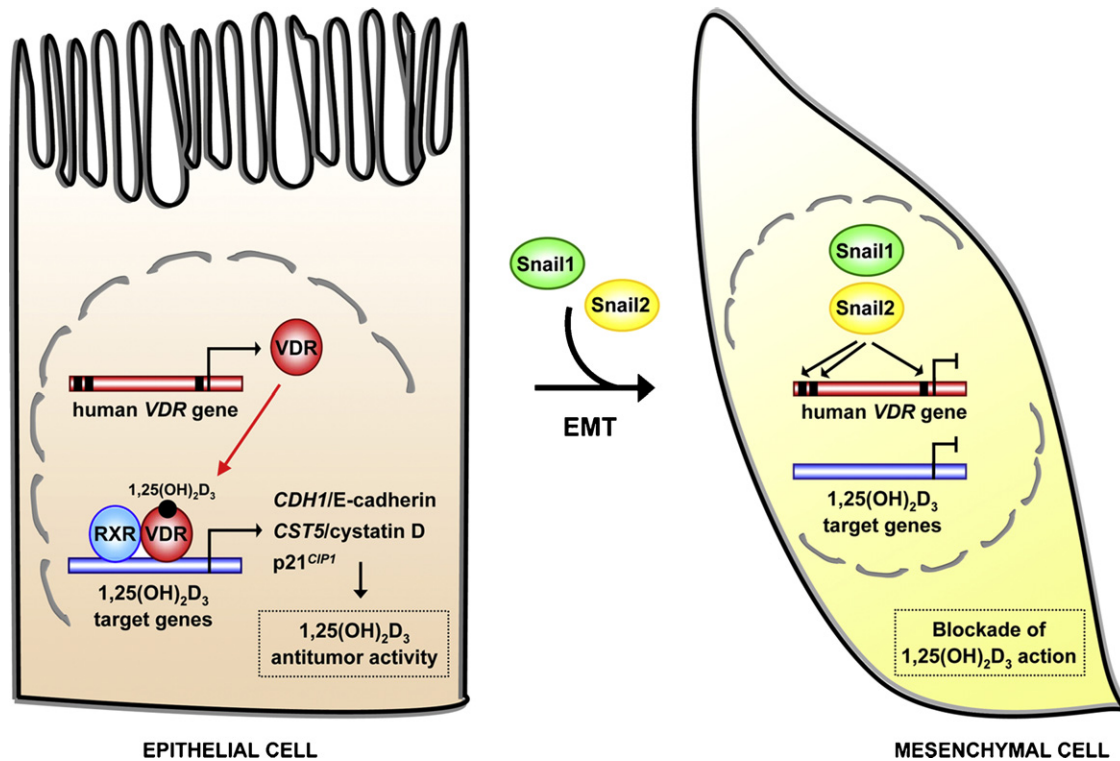
As ligand-activated *VDR* induces epithelial differentiation and the expression of *CDH1*/E-cadherin and other intercellular adhesion genes, *VDR* repression by Snail1 and Snail2 guarantees the induction of EMT even in the presence of  $1,25(\text{OH})_2\text{D}_3$ . This effect seems to be specific to the Snail family of transcription factors, since other EMT inducers such as Zeb1, Zeb2, E47 and Twist1 do not inhibit human *VDR* gene promoter [14].

### 5. Snail1 and Snail2 are responsible for *VDR* downregulation in colon cancer

To confirm the importance of Snail transcription factors in *VDR* downregulation during colon cancer progression, we analyzed *SNAIL1*, *SNAIL2* and *VDR* RNA expression in normal and tumoral biopsies from approximately one hundred colon cancer patients. We found that *SNAIL1* RNA was not present in any normal colonic tissue but it was expressed in 60–70% (depending on the study) of colon tumors. *VDR* expression in tumors was reduced with respect to that detected in the corresponding normal tissue in a similar percentage of tumors. In contrast, basal *SNAIL2* RNA expression was detected in most normal tissues and was also upregulated in 60% of colon tumors. Importantly, we observed that the overexpression of either *SNAIL1* or *SNAIL2* in individual tumors correlates with *VDR* downregulation [13,14,18,19]. In addition, we found that *SNAIL1* and *SNAIL2* RNA expression correlate directly and, therefore, a high percentage of tumors (42%) express both transcription factors. Remarkably, *VDR* downregulation was stronger in the tumors that express both *SNAIL1* and *SNAIL2* than in those that express only one of these genes. This is consistent with the additive effect exerted by Snail1 and Snail2 on *VDR* gene promoter in cultured human colon cancer cells [14]. Therefore, it seems that Snail1 and Snail2 collaborate in the repression of *VDR* gene in human colon cancer.



**Fig. 1.** The overexpression of Snail1 or Snail2 blocks the acquisition of the epithelial differentiated phenotype induced by  $1,25(\text{OH})_2\text{D}_3$  in SW480-ADH human colon cancer cells. Representative phase-contrast images (upper panels) and confocal laser immunofluorescence images showing  $\beta$ -tubulin staining (lower panels) of Mock, Snail1- and Snail2-overexpressing SW480-ADH cells treated with 100 nM  $1,25(\text{OH})_2\text{D}_3$  or vehicle for 48 h. Scale bar, 25  $\mu\text{m}$  (upper panels) and 10  $\mu\text{m}$  (lower panels).



**Fig. 2.** Scheme showing the repression of VDR and thereby of  $1,25(\text{OH})_2\text{D}_3$  action during the EMT induced by Snail1 or Snail2. Normal colon epithelial cells express VDR and are responsive to  $1,25(\text{OH})_2\text{D}_3$  target genes induction and antitumoral activity. Snail1 and/or Snail2 overexpression induces EMT through the regulation of several target genes. Among them, Snail factors bind to three E-boxes present in human VDR gene promoter and inhibit its expression, thus blocking  $1,25(\text{OH})_2\text{D}_3$  action.

Based on the hypothesis that tumor cells can influence adjacent normal cells and that some genetic alterations occur before the development of histologically abnormal tissue, we have recently expanded our studies to analyze the effect of tumoral *SNAIL1* overexpression on the levels of *VDR* in the histologically normal colonic tissue adjacent to the tumor. We have found that tumoral *SNAIL1* overexpression correlates with *VDR* downregulation both in the tumor and in the normal adjacent tissue, indicating that *SNAIL1* induces the release of paracrine signals from the tumoral cells that affect adjacent normal cells [20]. This could extend the unresponsiveness to vitamin D compounds to the normal tissue surrounding the tumor.

The repression of *VDR* by Snail transcription factors is not exclusive to colon cancer, as it has also been observed in human breast cancer cells (Ref. [21] and unpublished results). Also, Snail1 inhibits *VDR* expression in mouse osteoblasts [22]. As *SNAIL1* and/or *SNAIL2* upregulation has been reported in several types of cancer [16], this mechanism could explain the resistance to  $1,25(\text{OH})_2\text{D}_3$  action due to the *VDR* repression found in other types of tumors [10].

## 6. Conclusion

Our studies indicate that the transcription factors Snail1 and Snail2 are repressors of *VDR* and thus of  $1,25(\text{OH})_2\text{D}_3$  action in colon cancer cells (Fig. 2). Data from colon cancer biopsies indicate that *SNAIL1* and *SNAIL2* may be responsible for *VDR* downregulation during colon cancer progression.

Colon cancer patients with high levels of *SNAIL1* and *SNAIL2* have lower *VDR* expression and, therefore, will be resistant to a therapy with vitamin D compounds. Thus, tumor expression of *SNAIL1* and *SNAIL2* may be used as an indicator of patient adequacy to this treatment. Several groups have detected tumor-associated

RNA in the serum/plasma of cancer patients [23]. If tumor overexpression of *SNAIL1* and *SNAIL2* could be monitored by RT-PCR analysis of the RNA present in the serum/plasma, it would constitute a non-invasive method to select suitable patients for therapy with vitamin D compounds.

## Acknowledgements

We thank Toffa Evans and Robin Rycroft for their help with the English manuscript. The work in authors' laboratories is supported by the Ministerio de Ciencia e Innovación of Spain (SAF2007-60341, ISCIII-RETIC RD06/0020/0009 and RD06/0020/0020), Comunidad de Madrid (S-GEN-0266/2006) and European Union (MRTN-CT-2005-019496, NucSys).

## References

- [1] K.K. Deeb, D.L. Trump, C.S. Johnson, Vitamin D signalling pathways in cancer: potential for anticancer therapeutics, *Nat. Rev. Cancer* 7 (2007) 684–700.
- [2] G. Eelen, C. Gysemans, L. Verlinden, E. Vanoirbeek, P. De Clercq, D. Van Haver, C. Mathieu, R. Bouillon, A. Verstuyf, Mechanism and potential of the growth-inhibitory actions of vitamin D and analogs, *Curr. Med. Chem.* 14 (2007) 1893–1910.
- [3] H.G. Pálmer, J.M. González-Sancho, J. Espada, M.T. Berciano, I. Puig, J. Baulida, M. Quintanilla, A. Cano, A. García de Herreros, M. Lafarga, A. Muñoz, Vitamin  $\text{D}_3$  promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of  $\beta$ -catenin signaling, *J. Cell Biol.* 154 (2001) 369–387.
- [4] H.G. Pálmer, M. Sánchez-Carbayo, P. Ordóñez-Morán, M.J. Larriba, C. Cerdón-Cardó, A. Muñoz, Genetic signatures of differentiation induced by  $1\alpha, 25$ -dihydroxyvitamin  $\text{D}_3$  in human colon cancer cells, *Cancer Res.* 63 (2003) 7799–7806.
- [5] S. Álvarez-Díaz, N. Valle, J.M. García, C. Peña, J.M. Freije, V. Quesada, A. Astudillo, F. Bonilla, A. López-Otín, A. Muñoz, Cystatin D is a candidate tumor suppressor gene induced by vitamin D in human colon cancer cells, *J. Clin. Invest.* 119 (2009) 2343–2358.
- [6] H.S. Cross, E. Bajna, G. Bises, D. Genser, E. Kállay, R. Pötzi, E. Wenzl, F. Wrba, R. Roka, M. Peterlik, Vitamin D receptor and cytokeratin expression may

- be progression indicators in human colon cancer, *Anticancer Res.* 16 (1996) 2333–2337.
- [7] S.R.T. Evans, J. Nolla, J. Hanfelt, M. Shabahang, R.J. Nauta, I.B. Shchepotin, Vitamin D receptor expression as a predictive marker of biological behavior in human colorectal cancer, *Clin. Cancer Res.* 4 (1998) 1591–1595.
- [8] H.S. Cross, E. Kállay, M. Khorchide, D. Lechner, Regulation of extrarenal synthesis of 1,25-dihydroxyvitamin D<sub>3</sub>-relevance for colonic cancer prevention and therapy, *Mol. Aspects Med.* 24 (2003) 459–465.
- [9] D. Matusiak, G. Murillo, R.E. Carroll, R.G. Mehta, R.V. Benya, Expression of vitamin D receptor and 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase in normal and malignant human colon, *Cancer Epidemiol. Biomarkers Prev.* 14 (2005) 2370–2376.
- [10] M.G. Anderson, M. Nakane, X. Ruan, P.E. Kroeger, J.R. Wu-Wong, Expression of VDR and CYP24A1 mRNA in human tumors, *Cancer Chemother. Pharmacol.* 57 (2006) 234–240.
- [11] M.J. Campbell, L. Adorini, The vitamin D receptor as a therapeutic target, *Expert Opin. Ther. Targets* 10 (2006) 735–748.
- [12] K. Köstner, N. Denzer, C.S.L. Müller, R. Klein, W. Tilgen, J. Reichrath, The relevance of vitamin D receptor (VDR) gene polymorphisms for cancer: a review of the literature, *Anticancer Res.* 29 (2009) 3511–3536.
- [13] H.G. Pálmer, M.J. Larriba, J.M. García, P. Ordóñez-Morán, C. Peña, S. Peiró, I. Puig, R. Rodríguez, R. de la Fuente, A. Bernad, M. Pollán, F. Bonilla, C. Gamallo, A. García de Herreros, A. Muñoz, The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer, *Nat. Med.* 10 (2004) 917–919.
- [14] M.J. Larriba, E. Martín-Villar, J.M. García, F. Pereira, C. Peña, A. García de Herreros, F. Bonilla, A. Muñoz, Snail2 cooperates with Snail1 in the repression of vitamin D receptor in colon cancer, *Carcinogenesis* 30 (2009) 1459–1468.
- [15] M.J. Larriba, N. Valle, H.G. Pálmer, P. Ordóñez-Morán, S. Álvarez-Díaz, K.F. Becker, C. Gamallo, A. García de Herreros, J.M. González-Sancho, A. Muñoz, The inhibition of Wnt/beta-catenin signalling by 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> is abrogated by Snail1 in human colon cancer cells, *Endocr. Relat. Cancer* 14 (2007) 141–151.
- [16] H. Peinado, D. Olmeda, A. Cano, Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat. Rev. Cancer* 7 (2007) 415–428.
- [17] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, *J. Clin. Invest.* 119 (2009) 1420–1428.
- [18] C. Peña, J.M. García, J. Silva, V. García, R. Rodríguez, I. Alonso, I. Millán, C. Salas, A. García de Herreros, A. Muñoz, F. Bonilla, E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations, *Hum. Mol. Genet.* 14 (2005) 3361–3370.
- [19] C. Peña, J.M. García, V. García, J. Silva, G. Domínguez, R. Rodríguez, C. Maximiano, A. García de Herreros, A. Muñoz, F. Bonilla, The expression levels of the transcriptional regulators p300 and CtBP modulate the correlations between SNAIL, ZEB1, E-cadherin and vitamin D receptor in human colon carcinomas, *Int. J. Cancer* 119 (2006) 2098–2104.
- [20] C. Peña, J.M. García, M.J. Larriba, R. Barderas, I. Gómez, V. García, J. Silva, G. Domínguez, R. Rodríguez, J. Cuevas, A. García de Herreros, J.I. Casal, A. Muñoz, F. Bonilla, *SNAIL* expression in colon cancer related with *CDH1* and *VDR* downregulation in normal adjacent tissue, *Oncogene* 28 (2009) 4375–4385.
- [21] M.K. Mittal, J.N. Myers, S. Misra, C.K. Bailey, G. Chaudhuri, *In vivo* binding to and functional repression of the *VDR* gene promoter by SLUG in human breast cells, *Biochem. Biophys. Res. Commun.* 372 (2008) 30–34.
- [22] C.A. de Frutos, R. Dacquin, S. Vega, P. Jurdic, I. Machuca-Gayet, M.A. Nieto, Snail1 controls bone mass by regulating *Runx2* and *VDR* expression during osteoblast differentiation, *EMBO J.* 28 (2009) 686–696.
- [23] J.C.H. Tsang, Y.M.D. Lo, Circulating nucleic acids in plasma/serum, *Pathology* 39 (2007) 197–207.