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Review

The dexamethasone induced osteogenic differentiation of dental follicle cells

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Summary. Mesenchymal stem cells are excellent for in vitro studies about biological processes during the differentiation of osteogenic progenitor cells into mineralizing cells such as osteoblasts. Human dental follicle cells (DFCs) are dental mesenchymal stem cells and they can be isolated from third molar teeth. Because DFCs are the genuine progenitor cells of periodontal tissue cells, they have been used for the evaluation of molecular mechanisms during the differentiation of undifferentiated stem cells into alveolar osteoblasts and cementoblasts. To reveal molecular mechanisms of osteogenic differentiation, initial studies investigated the proteome and the transcriptome of DFCs after the induction of the osteogenic differentiation with the glucocorticoid dexamethasone. These studies showed for example that dexamethasone induces the transcription factor ZBTB16 (zinc finger and BTB domain containing protein 16) and that ZBTB16 is crucial for osteogenic differentiation of DFCs. This article is a survey of the molecular mechanisms in DFCs during osteogenic differentiation with dexamethasone.

Key words: Dental follicle cells, Osteogenic differentiation, Transcription factors, Signaling pathways, Periodontium

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Introduction

Bone and teeth are derived from different embryonic tissues. While most of the skeleton originates from the paraxial and lateral plate mesoderm, facial bone and mineralizing tissues of the dental mesoderm (dentin, cementum, alveolar bone) derive from neural crest cells, which are originally from the embryonic ectoderm (Robey, 2011; Bianco and Robey, 2015). Dental mesodermal stem/progenitor cells are the origins of the dental pulp/dentin complex and of all tooth root tissues (Diekwisch, 2001). The mineralizing tissues of the tooth attachment apparatus are dentin, the dental cementum and the alveolar bone (Huang et al., 2009; Morsczeck et al., 2013). The dental cementum and the alveolar bone of the tooth root are derived from cells of the dental follicle, which is a tooth germ tissue comprising stem/progenitor cells (DFCs) for alveolar bone osteoblasts and for cementoblasts (Diekwisch, 2001; Dangaria et al., 2011; Gao et al., 2016). In previous studies we have isolated DFCs from the human dental follicle (Morsczeck et al., 2005, 2010). These isolated DFCs differentiate for example into jaw bone osteoblast like cells under in vitro conditions and they are useful for in vitro studies about the osteogenic differentiation of dental progenitor cells (Morsczeck and Schmalz, 2010; Honda et al., 2010; Morsczeck, 2015). We consider that a profound understanding of the sophisticated molecular mechanism of the osteogenic differentiation is required for a successful regeneration of dental tissues with dental stem cells. To find this molecular mechanism of osteogenic differentiation, our initial studies with DFCs investigated molecular processes after the osteogenic differentiation with a standard cell culture medium. This cell culture medium contains glucocorticoid

dexamethasone for the induction of the differentiation. Our previous studies utilized proteomics and transcriptomics to reveal regulated genes and proteins in DFCs (Morsczeck et al., 2009a,b; Morsczeck and Schmalz 2010; Saugspier et al., 2010). Our proteomics and transcriptomics results and our following investigations considered the molecular processes in DFCs, which are probably different to that of other osteogenic progenitor cells (Morsczeck and Schmalz, 2010b; Silvério et al., 2012; James, 2013; Felthaus et al., 2014a,b).

One postulated dexamethasone induced mechanism for the ostoegenic differentiation in DFCs will be presented and discussed in more details in our review article. However, we have to mention an important review article about the effects of dexamethasone on the osteogenic differentiation of stem cells before. Here, the authors postulated a particular dexamethasone induced signaling pathway that mediates the differentiation of mesenchymal stem cells or other types of osteogenic progenitor cells into mineralizing osteoblasts (Langenbach and Handschel, 2013). This article put the osteogenic transcription factor RUNX2 in a central position. For the initiation of the differentiation the glucocorticoid dexamethasone induces the expression of the following genes for the differentiation: FHL2 (Four and a half LIM domains protein 2), MKP-1 (also known as DUSP1; Dual specificity protein phosphatase 1) and TAZ (Tafazzin). These proteins stimulate either the transcription of the messenger RNA of RUNX2 (FHL2) or they activate the RUNX2 protein via a) phosphorylation (MKP-1) or b) direct protein-protein interactions (TAZ) (Langenbach and Handschel, 2013). This postulated mechanism for the osteogenic differentiation is based on the results of various independent studies with different types of osteogenic stem/progenitor cells and Langenbach and Handschel did not distinguish between osteogenic progenitor cells from different origins in their review article, for example between bone marrow derived mesenchymal stem cells (MSCs) and neural crest cell derived osteogenic progenitor cells. Intriguingly, almost none of these genes, which are mentioned in their review article, were significantly induced in neural crest cell derived DFCs after the induction of osteogenic differentiation with dexamethasone (Morsczeck et al., 2009a,b; Morsczeck and Schmalz, 2010a; Saugspier et al., 2010; Felthaus et al., 2014a). Actually, we identified a number of genes such as the transcription factor zinc finger and BTB domain containing protein 16 (ZBTB)-16, which are frequently up-regulated during the differentiation with dexamethasone, but, unfortunately, not considered by Langenbach and Handschel. However, we suppose that at least ZBTB16 has to be considered for the osteogenic differentiation of all types of mesenchymal stem cells, because a previous study showed that this transcription factor is involved in the differentiation of bone marrow derived mesenchymal stem cells (Ikeda et al., 2005).

Our article therefore summarizes our current knowledge about ZBTB16 and the dexamethasone-based osteogenic differentiation in DFCs. Nonetheless even if it is only piecework and impossible to reconstruct a reasonable signaling pathway, this article offers otherwise different suggestions for future studies on molecular mechanisms of the dexamethasone-based differentiation of DFCs.

The ZBTB16 dependent osteogenic differentiation

Transcription factors play a decisive role for the biological processes of osteogenic differentiation in DFCs (Viale-Bouroncle et al., 2013b). We investigated gene expression profiles of transcription factors during the osteogenic differentiation of DFCs in dexamethasone (Morsczeck et al., 2005; Morsczeck, 2006; Morsczeck and Schmalz, 2010; Saugspier et al., 2010). These studies showed that dexamethasone induces the transcription factor ZBTB16, which belongs to the Krüppel-like zinc finger type transcription factors (Zhang et al., 1999). However, well known osteogenic transcription factors such as RUNX2 or OSTERIX were not induced in DFCs (Morsczeck, 2006; Morsczeck et al., 2009b). ZBTB16 is a glucocorticoid response gene and it is involved in a number of biological processes such as glucocorticoid-induced apoptosis in lymphocytes (Schmidt et al., 2006; Wasim et al., 2010). In DFCs, ZBTB 16 is expressed only in the presence of the glucocorticoid dexamethasone (Morsczeck and Schmalz 2010). It is neither expressed in naïve DFCs nor in DFCs after cultivation in osteogenic differentiation media without dexamethasone; for example in osteogenic differentiation media with the bone morphogenetic protein (BMP)-2 or with the insulin like growth factor (IGF)-2 (Saugspier et al., 2010).

While a positive feedback loop between the BMP-signaling pathway and the transcription factor DLX3 is involved in the osteogenic differentiation of DFCs (Viale-Bouroncle et al., 2012, 2015; Morsczeck, 2015), we suppose an additional alternative and dexamethasone induced differentiation pathway. For this pathway ZBTB16 takes an important part (Felthaus et al., 2014a,b). ZBTB16 is generally involved in limb and axial skeletal patterning (Barna et al., 2005). Inoue et al., showed that ZBTB16 was up-regulated in mesenchymal stem cells from patients suffering from ossification of the posterior longitudinal ligament (Inoue et al., 2006). In bone marrow derived mesenchymal stem cells, ZBTB16 acts upstream of the osteogenic transcription factor Runt-related transcription factor (RUNX)-2 and it is involved in the expression of genes of the extra cellular matrix proteins collagen type I (COLI) and osteocalcin (OCN) and of the alkaline phosphatase (ALP) (Ikeda et al., 2005). We showed that neither dexamethasone nor ZBTB16 induce the expression of RUNX2 in DFCs and that RUNX2 gene silencing did not prevent the osteogenic differentiation of DFCs

(Saugspier et al., 2010; Felthaus et al., 2014a,b). Moreover, ZBTB16 binds directly to the promoter regions of the gene of the transcription factor NR4A3, which is highly induced in DFCs after osteogenic differentiation (Morsczeck et al., 2009b), but it does not bind to the promoter region of the RUNX2 gene (Felthaus et al., 2014a). These results suggest a RUNX2-independent ZBTB16 pathway for osteogenic differentiation in DFCs, which is induced only by dexamethasone (Saugspier et al., 2010; Felthaus et al., 2014a). Interestingly, Onizuka et al., suggest that ZBTB16 could also be part of the osteogenic differentiation of periodontal ligament (PDL) stem cells (Onizuka et al., 2016). Here, the expression of ZBTB16 correlates with the expression of a number of osteogenic differentiation markers. Nonetheless, there are also differences between DFCs and PDL stem cells. While ZBTB16 is a downstream target gene of the transcription factor OSTERIX in PDL stem cells (Onizuka et al., 2016), our data suggest that OSTERIX is vice versa a target gene of ZBTB16. However, in both DFCs and PDL stem cells the expression of ZBTB16 is required for the expression of the osteogenic differentiation marker OCN (Felthaus et al., 2014b; Onizuka et al., 2016). Furthermore we showed that ZBTB16 induces the expression of the glycoprotein Stanniocalcin (STC)1, which regulates calcium and phosphate balance in the body (Felthaus et al., 2014b). STC1 is possibly regulated via a positive feedback loop with ZBTB16 in DFCs. However, we only have a vague speculation on the actual molecular mechanism downstream of ZBTB16. We consider for example that STC1 influences the process of osteogenic differentiation via phosphate transport, which is important for mineralization (Johnston et al., 2010). However, based upon these results it is not possible to draw final conclusions on the mechanism of a RUNX2-independent osteogenic differentiation in DFCs. We consider that genes/proteins, which are up-regulated after the overexpression of ZBTB16 in DFCs, are involved in the dexamethasone induced differentiation in DFCs. Two ZBTB16 induced genes are discussed in the next section of this review article.

Osteogenic differentiation and the part of the extracellular proteins parathyroid hormone related peptide (PTHrP) and osteocalcin (OCN)

One target gene of ZBTB16 in DFCs is the gene of the parathyroid hormone related peptide (PTHrP, also known as PTHLH), which is highly associated with bone development and differentially expressed after ZBTB16 overexpression (Felthaus et al., 2014b). PTHrP is an extracellular protein and required for both the regulation of enchondral bone development and the differentiation of bone precursor cells during the development of craniofacial tissues (Kronenberg, 2006; Pan et al., 2013). For example, during enchondral ossification it keeps chondrocytes proliferating and delays the differentiation into hypertrophic chondrocytes, which is essential for the length growth of bone (Vortkamp, 2000; Ehlen et al., 2006; Lenton et al., 2011). PTHrP is furthermore involved in cell cycle regulation in different tissue cell types (Kronenberg, 2006; Miao et al., 2008) and it is also required for tooth eruption after activation of the alveolar bone resorption (Philbrick et al., 1998; Rakian et al., 2013). Here, PTHrP, which is secreted from cells of the dental follicle, attracts and/or activates osteoclasts in the dental crypt (Nakchbandi et al., 2000). A recent study investigated the influence of PTHrP on the osteogenic differentiation of DFCs (Klingelhöffer et al., 2016a). Here, DFCs secreted PTHrP after the induction of osteogenic differentiation with dexamethasone (Klingelhöffer et al., 2016a). Similarly to the differentiation with dexamethasone, the extracellular concentration of PTHrP increased after the induction of osteogenic differentiation with BMP2 (Morsczeck et al., 2017). However, the supplementation of the osteogenic differentiation medium with the PTHrP protein inhibited osteogenic differentiation, but gene silencing of PTHrP neither supported nor inhibited the differentiation of DFCs (Klingelhöffer et al., 2016a). We believe that ZBTB16 induces the secretion of PTHrP and that the extracellular PTHrP concentration regulates osteogenic differentiation in DFCs. At the moment, hovewer, the molecular mechanisms remain elusive for the regulation of both PTHrP protein secretion and for the inhibition of the osteogenic differentiation down-stream of PTHrP. It is very likely that PTHrP inhibits the dexamethasone-induced differentiation of DFCs downstream of ZBTB16 via a negative feedback loop. Interestingly, a previous study showed that typical target proteins of the PTHrP pathway such as protein kinase A support BMP2-induced osteogenic differentiation in DFCs (Viale-Bouroncle et al., 2015). However, we suppose that the PTHrP pathway and especially protein kinase PKA play a different (probably inhibitory) role for the ZBTB16-depedendent osteogenic differentiation in DFCs, but additional sophisticated investigations are required to prove this hypothesis.

Osteocalcin (OCN) is the most abundant non-collagenous protein in bone and we showed previously that its expression can be induced slightly by ZBTB16 overexpression in DFCs (Felthaus et al., 2014b; Brennan-Speranza and Conigrave, 2015). The presence of OCN in cell culture medium is generally a reliable marker for the induction of the osteogenic differentiation of mesenchymal stem cells under in vitro conditions (Nakamura et al., 2009). However, we showed a constitutive expression and secretion of the OCN protein, which is also independent from the induction of osteogenic differentiation (Klingelhöffer et al., 2016b). Here, surprisingly, the extracellular concentrations of OCN were similar in cell cultures with the standard cell culture medium and with the osteogenic differentiation medium. However, more astonishing, after 4 weeks the OCN level in the osteogenic differentiation medium decreased, while the OCN concentration in the control

medium was unchanged. At this point in time DFCs formed mineralized structures with OCN (Klingelhöffer et al., 2016b). We suppose that soluble OCN proteins in the cell culture medium bind to the mineralizing collagenous matrix at this point of time during the differentiation (Klingelhöffer et al., 2016b). This is possibly the reason for the decreasing extracellular OCN level in the cell culture medium at the time of biomineralization. The constitutive expression of the OCN protein in DFC cell cultures may also explain the missing induction of RUNX2 during the differentiation of DFCs. It is because the activity of the RUNX2 transcription factor controls the promoter activity of the OCN (BGLAP) gene after the induction of the osteogenic differentiation (Hassan et al., 2004; Franceschi et al., 2007). We believe that an induction of the RUNX2 expression is not required for the dexamethasone-based osteogenic differentiation of DFCs, because OCN is almost constitutively expressed or even down-regulated during the osteogenic differentiation (Saugspier et al., 2010). Furthermore we believe that ZBTB16 sustains the expression of OCN in DFCs during the differentiation with dexamethasone (Felthaus et al., 2014b). However, this conclusion is only a vague hypothesis for further investigations, because it does not consider a number of osteogenic differentiation markers, which are also target genes of RUNX2 (Franceschi et al., 2007).

Conclusion and outlook

ZBTB16 protein takes part in a number of biological processes and it very often has the molecular function of a DNA binding protein (Suliman et al., 2012). Nonetheless, the actual molecular function of the ZBTB16 protein in osteogenic differentiation remains elusive. Our previous studies showed that ZBTB16 induces a number of genes or gene products and that ZBTB16 binds to gene promoters of some of these target genes including the NR4A3 gene (Viale-Bouroncle et al., 2012). However, it is still not clear how ZBTB16 is involved in the gene expression and protein secretion for example of OCN and PTHrP. A qRT-PCR array revealed that not only the gene expression of OCN (also known as BGLAP) but also the gene expression of PTHrP decreased after the induction of the osteogenic differentiation with dexamethasone (Fig. 1), while the protein expression of PTHrP and OCN was increased or at least constitutively expressed (Klingelhöffer et al., 2016a,b). Interestingly, BMP2 induces the gene expression of PTHrP and OCN (Fig. 1), so their gene expression is positively correlated with the induction of the osteogenic differentiation of DFCs. Because of these contradicting data, we suppose that ZBTB16 has more sophisticated molecular mechanisms than that of a DNA binding protein (transcription factor). Interestingly, it is already known that ZBTB16 is also involved in the post

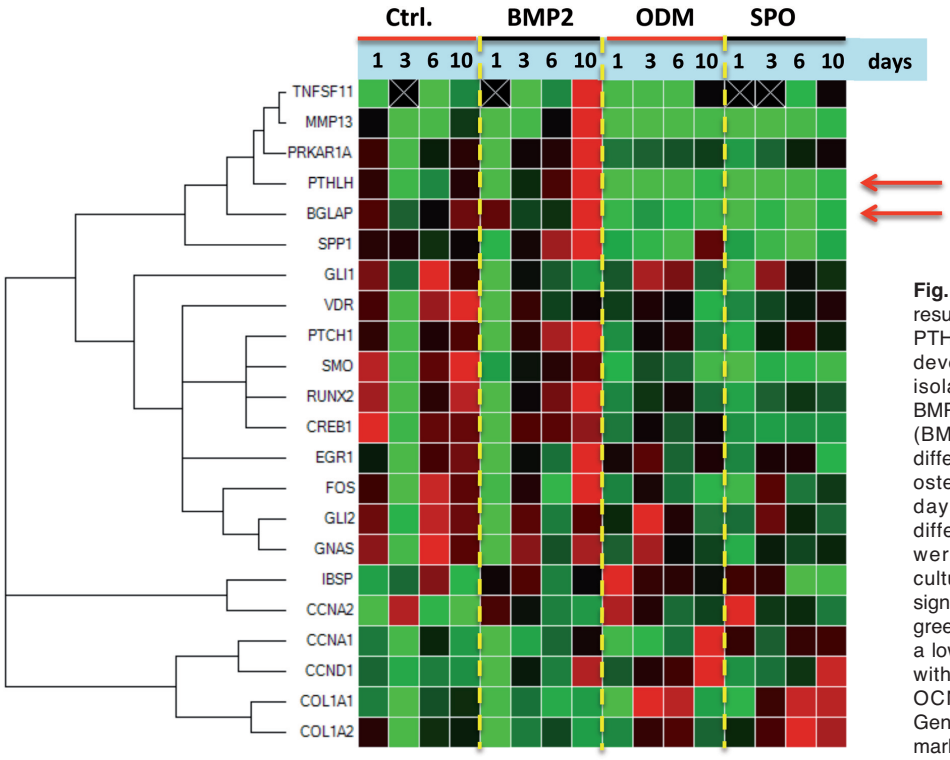

Fig. 1. Clustergram of real-Time RT-PCR Array results with the "Development - Hedgehog and PTH signaling pathways in bone and cartilage development" PCR array. Total RNAs were isolated from DFCs in long-term cultures in BMP2 based osteogenic differentiation medium (BMP2), dexamethasone based osteogenic differentiation medium (ODM) and StemPro® osteogenic differentiation medium (SPO) at days 1, 3, 6 and 10 after the induction of differentiation. For control (Ctrl.) total RNAs were isolated from DFCs in standard cell culture medium. In the clustergram red tiles signify a high gene expression, black/grey and green tiles show a middle gene expression and a low gene expression, respectively. Black tiles with a cross designate no gene expression. OCN and PTHrP are marked with arrows. Genes BGLAP (OCN) and PTHLH (PTHrP) are marked with arrows.

translational regulation of cytoplasmic proteins (Suliman et al., 2012; Zhang et al., 2015). Recently, for example, Yuan and co-workers showed that ZBTB16 in a complex with other cytoplasmic proteins mediates the specific protein degradation of ATG14L, which is an autophagy related protein, by ubiquitination. This degradation of ATG14L regulates the initiation of autophagy, which is crucial for the degradation of misfolded proteins in neural tissue cells (Zhang et al., 2015). The regulation of autophagy could help us to understand the protein expression and/or the protein secretion of PTHrP and/or of OCN in DFCs during the differentiation with dexamethasone. Interestingly, the biological process of autophagy is currently associated with the induction of apoptosis in tooth germ tissue cells during tooth development (Yang et al., 2013a,b). Here tooth germ cells from the dental follicle or from the dental papilla express the authophagy protein LC3, which is a mammalian homolog of the yeast autophagy related gene Atg8. Moreover, double- immunofluorescence analysis revealed the partial co-localization of LC3 and TUNEL, which is a specific marker for apoptosis (Yang et al., 2013a,b). Interestingly, a number of genes, which are associated with the induction of apoptosis, are differentially expressed in DFCs during osteogenic differentiation (Morsczeck et al., 2009b; Saugspier et al., 2010; Viale-Bouroncle et al., 2012, 2013a); (Fig. 2). Moreover, previous studies showed that ZBTB16 interferes with the induction of apoptosis in different cell lines (Wasim et al., 2010) and we suppose that both the expression the apoptosis protein caspase 3 (Miura et al., 2004; Larsen et al., 2010) and the induction of autophagy (Kim et al., 2016; Li et al., 2016) are required for the differentiation of somatic stem cells. So we believe that at least parts of these biological processes have to be considered for further investigations about

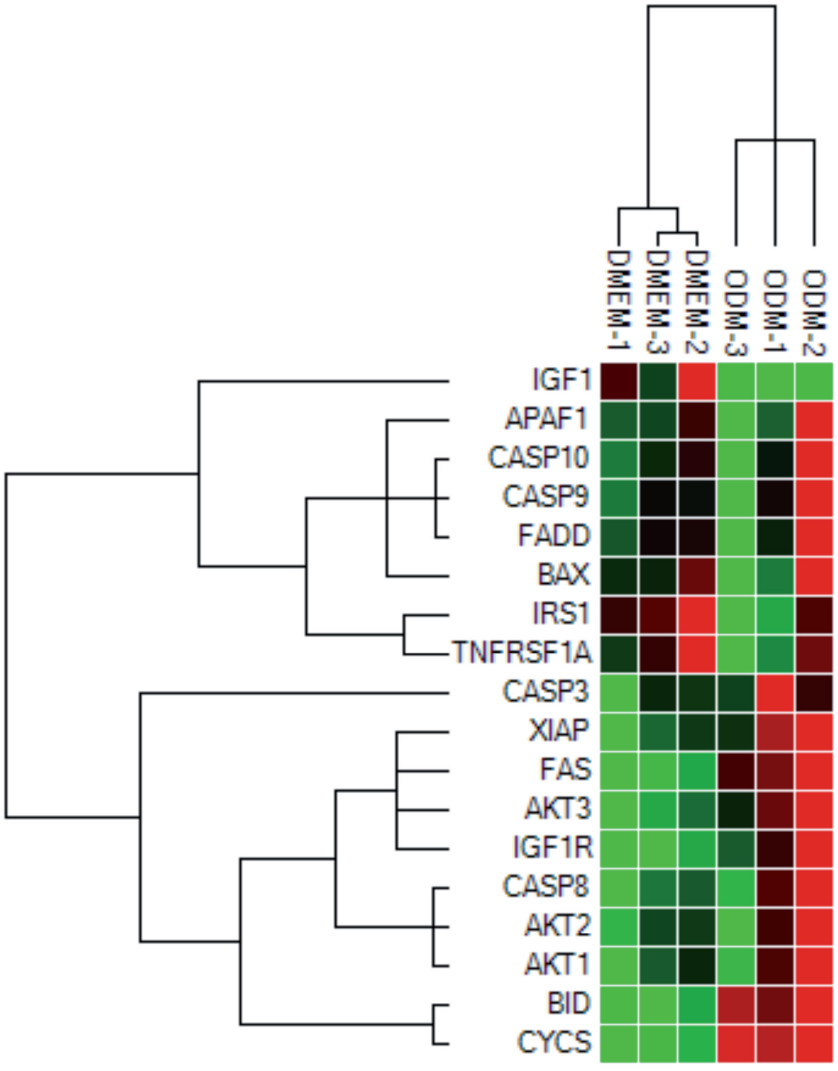

Fig. 2. Clustergram of real-Time RT-PCR Array results with the "Apoptosis and survival - Caspase cascade" PCR array. Total RNAs were isolated from DFCs in long-term cultures in dexamethasone based osteogenic differentiation medium (ODM) at day 1 after the induction of the differentiation. For control (Ctrl.) total RNAs were isolated from DFCs in standard cell culture medium.

ZBTB16 and the osteogenic differentiation of DFCs.

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