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Effects of Strontium on Human Osteoblasts Extracted from a Cleidocranial Dysplasia Patient – In Vitro Study

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1. INTRODUCTION

1.1. Cleidocranial Dysplasia

Cleidocranial Dysplasia syndrome is known by different names such as Marie Sainton syndrome, Mutational Dysostosis, Cleidocranial Dysostosis and Dysostosis Cleidocranialis [(1)]. *Cleidocranial Dysplasia* was described for the first time in the 19th century by Gustav Scheuthauer, Pierre Marie and Paul Sainton[(2, 3)] who reported the two most striking manifestations, viz. the hypo- or aplastic clavicles and the abnormal, excessive number of teeth ranging up to 70 teeth in some cases [(4)]. Since then, over 1000 cases from the whole world with a wide range of clinical manifestations have been documented in medical literature [(5-7)].

In many medical references the abbreviation (CCD), which will be also used often in this research, has been used to describe this genetic disease.

In 1908 the Swedish physician Hultkranz described this as 'quatermoon' physiognomy: 'The nasion-alveolar line appears more upright than usual (…) if a protruding forehead and the strong prognathic mandible is added, the result is a concave facial profile, a quatermoon physiognomy' [(8)].

Cleidocranial Dysplasia is notable for aplasia or hypoplasia of the clavicles, characteristic craniofacial malformations, and the presence of numerous supernumerary and unerupted or impacted teeth [(9)]. Other clinical hallmarks of CCD include delayed or failure in the closure of cranial fontanels and sutures, in addition to other skeletal anomalies. Thus, CCD is not limited to the craniofacial region; it is defined by many medical references as a generalized dysplasia and skeletal disorder of the entire skeletal system [(10, 11)].

It is considered to be a rare genetic defect affecting mainly the membranous bone formation, with an incidence estimated at 1: 200,000 live births [(5)] and a prevalence of 1: 1,000,000 [(12)], affecting in most cases the skull and the clavicles. However, the prevalence of affecting other parts of the skeleton is still high.

1.1.1. Etiology and Pathogenesis

Cleidocranial Dysplasia is transmitted by an autosomal-dominant mode of inheritance with high penetrance and a different degree of expressivity affecting both skeletal and dental systems [13, 14]. A recessive form of this syndrome has been reported in two families. About one third of the cases show different and new mutations. The frequency of *Cleidocranial Dysplasia* is almost the same among males and females; and there is also no racial predilection.

Studies involving large population groups in South Africa have localized the origin of this disorder to the short arm of chromosome 6p21 and confirmed that the transcription factor *(Runx2)* is the causative factor of this genetic disorder [15, 16].

Most patients with this disorder are of normal intelligence [9], and until now there are no studies supporting any relation between CCD and a low IQ level. Many patients treated in the orthodontic clinic of Regensburg University Hospital, showed good intelligence and, in some cases managerial, work positions.

Intramembranous and endochondral bones in the skull are primary affected, resulting in a sagittally diminished cranial base, transverse enlargement of the calvarium, and delayed closure of the fontanels and sutures [17, 18]. Late closure of fontanels is also considered as a common pathological feature of other syndromes, such as Basal cell nevus syndrome and Crouzon's syndrome (Craniofacial syndrome), but together with other characteristic features, *Cleidocranial Dysplasia* can be easily differentially diagnosed [5, 9].

The biparietal and frontal bossing and the extension of the cranial vault result from the hydrocephalic pressure exerted on unossified regions of the skull, especially the fontanel areas. The deficiency or complete absence of the clavicles is responsible for the characteristic long appearance of the neck and the narrow shoulders. In general, the special facial appearance of CCD is a result of the underdeveloped and abnormal middle third of the face combined with the abnormalities in the dental alveolar complex [9, 17].

Many studies have supported the idea that delayed or failed eruption of the teeth is associated with a lack of cellular cementum. This failure of cementum formation is thought to be a direct result of the mechanical resistance to eruption due to dense alveolar bone overlying the unerupted teeth. On other hand, it has been

hypothesized, that the formation of supernumerary teeth might be due to incomplete or severely delayed resorption of the dental lamina which is reactivated at the time of crown completion of the normal permanent dentition [9]. However, until now there is no definitive explanation for this phenomenon.

1.1.2. Clinical Manifestations

The clinical appearance of *Cleidocranial Dysplasia* includes very characteristic and distinct features that make this syndrome very pathognomonic *(Fig.1)*. In general, the patients show a mildly to moderately shortened stature, with the neck appearing elongated and narrow and the shoulders markedly drooped or at least oblique at 45°, which results from absent or malformed clavicles [9]. About 10% of CCD patients have been recorded to show complete failure in the formation of the clavicles.

Hypermobility of the shoulders, which is very characteristic of CCD patients, is a result of complete or partial absence of clavicular calcification, with associated muscular defects. This enables the patients to show variable levels of approximation of the shoulders in the anterior plane [17, 19].

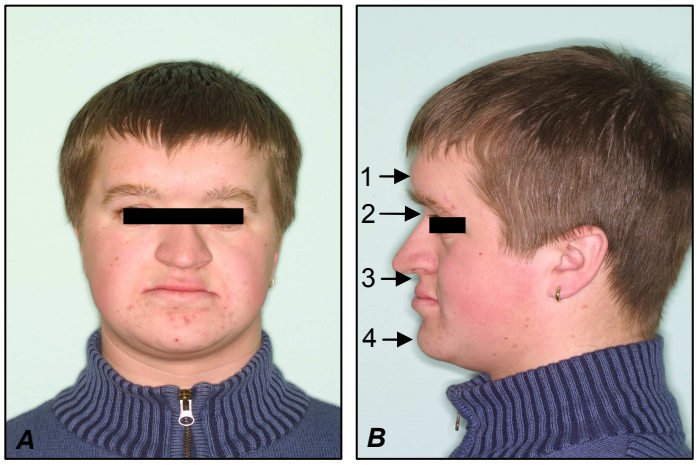


Fig.1. Extra-oral photos of 19 years old patient with CCD syndrome, who is being treated in the Orthodontic and Maxillofacial Surgery clinic in Regensburg University Hospital. *(A)* Shows somehow large head with broad based nose and poorly developed middle face. *(B)* Shows profile hallmarks of CCD: *1.* prominent forehead, *2.* Depressed nasal bridge, *3.* Maxillary hypoplasia, *4.* Mandibular progenie with prominent chin. *Regensburg University Hospital, Orthodontic Clinic Archive*

The head is large and brachycephalic with a persistent metopic (frontal) suture [20]. Most patients show pronounced frontal, parietal, and occipital bossing. The facial bones and paranasal sinuses are hypoplastic or aplastic, giving the face a small and short appearance. The nose is also affected. CCD patients show a broad based nose, with a depressed nasal bridge. Ocular hypertelorism had been recorded for this syndrome [21] *(Fig.1)*.

In general, the entire skeleton can be affected, with defects of the pelvis, long bones, and long fingers. Hemivertebrae and posterior wedging of the thoracic

vertebrae may contribute to the development of kyphoscoliosis and pulmonary complications [17] *(Fig.2)*.

The clinical manifestations associated with the CCD syndrome can be classified based on the affected region as follows: [10, 11, 22, 23]

TABLE 1. Summary of all possible affected regions in CCD with the common associated symptoms

Region	*Common associated symptoms*
Head	1. A large brachycephalic head. 2. A broad forehead with frontal, parietal or occipital bossing. 3. Delayed or failed closure of the fontanels and sutures. 4. Poorly developed mid-frontal area showing a frontal groove. 5. Soft skull in infancy.
Face	1. A depressed nasal bridge. 2. Hypertelorism with possible exophthalmos. 3. A small, flattened facial appearance (mid-face hypoplasia) with prognathic mandible (true or pseudo-progenia). 4. Vertical maxillary deficiency.
Shoulders and thorax	1. Ability to approximate the shoulders in the anterior plane. 2. Dimpling of the skin due to mild hypoplasia of the clavicles. 3. Sloping, almost absent shoulders secondary to severe hypoplasia or complete absence of the clavicles. 4. Narrow thorax which may lead to respiratory distress during early infancy.
Spine	1. Scoliosis, 2. Kyphosis.
Hands	1. Brachydactyly. 2. Short distal phalanges. 3. Tapering fingers. 4. Nail dysplasia or hypoplasia. 5. Short, broad thumbs. 6. Clinodactyly of the 5th fingers.
Other abnorma-lities	1. Hearing loss. 2. Abnormal gait. 3. Joint hypermobility. 4. Muscular hypotonia.

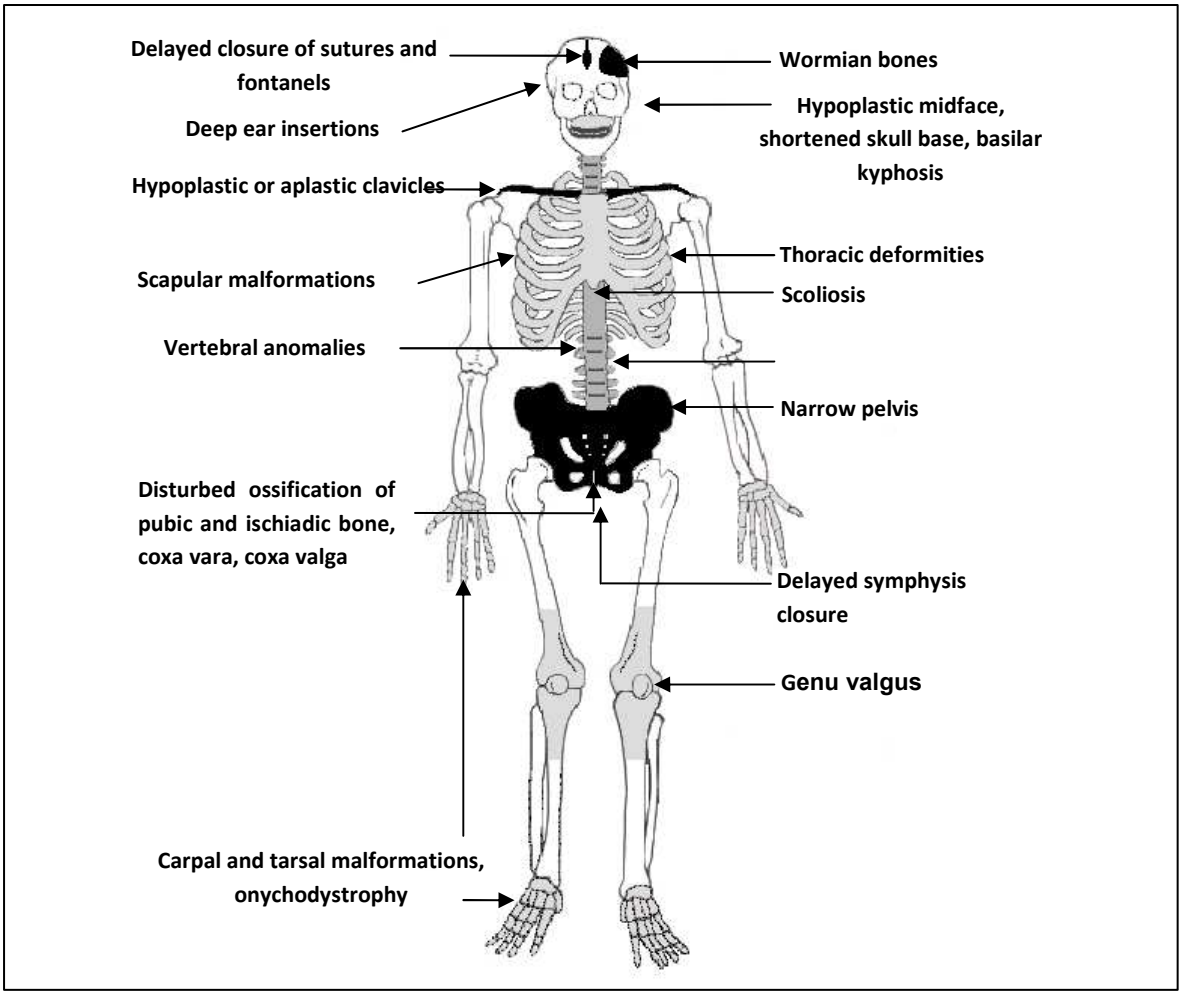


Fig.2. Schematic illustration of the major and minor affected regions in patients with cleidocranial dysplasia [24]. Note that most of the skeletal system may be affected with different defects; however, the severity of these defects is still highly variable. The areas with dark shading represent the sites most frequently affected with cleidocranial dysplasia mutational symptoms of high severity. Regions like the clavicles, cranial sutures, dentofacial complex and pelvis, with black shading, in most cases show a severe degree of defects and deformations.
On other hand, areas with light shading like the thoracic, vertebral column and the carpal and tarsal may show mild symptoms or, in some cases, may not show any form of deformations or functional defects.

1.1.3. Oral and Dental Manifestations

The underdeveloped (hypoplastic) maxilla leads to a relatively prognathic appearance of the mandible. However, some patients may exhibit different degrees of real or true mandibular prognathism, due to a considerable increase of lower jaw length in relation to a short cranial base. In most cases, the palate is narrow and highly arched, and there is an increased incidence of submucosal median clefts *(Fig.3)*.

Complete or partial clefts of the palate, which may involve both the hard and soft tissues, have also been diagnosed in many cases. In the lower jaw, delayed closure of the mandibular symphysis was reported by Scully and Cawson [20]. However, complete nonunion of the symphysis of the mandible was also found in some CCD patients [9].

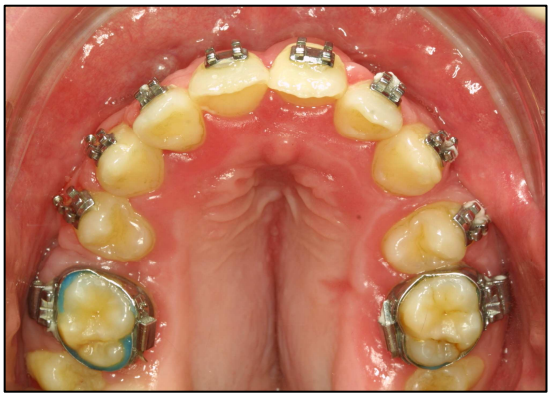


Fig.3. Intra-oral Photo of the same patient in Figure [1], same age also, demonstrating a submucosal median palatinal cleft. *Regensburg University Hospital, Orthodontic Clinic Archive*

The development, maturation, and eruption of the primary teeth are mostly normal. However, an extreme delay in the process of normal and expected root resorption of primary teeth occurs, which results in prolonged retention and late exfoliation of deciduous dentition. The physiologic eruption of the permanent dentition is severely delayed, and in many patients more than half of the teeth fail to erupt [19]. Adult individuals with *Cleidocranial Dysplasia* who show a mixed dentition (both primary and permanent teeth) in their oral cavity are a very common observation

Unerupted or retained supernumerary teeth are often present in all regions of both jaws, and mostly look like the premolars [9]. As many as 63 unerupted supernumerary teeth were documented in one patient [25]. These teeth develop by the time of completion of normal crown formation in the permanent dentition, just lingually and occlusally to the normal unerupted crown. In general, one supernumerary tooth per normal tooth is noted on average.

Severe malocclusion and crowding is often a characteristic dental finding in patients with this congenital syndrome. Many factors contribute to the malocclusion including over-retention of the deciduous (primary) teeth, failure of eruption of the permanent teeth, presence of numerous supernumerary teeth and maxillary hypoplasia. In most cases, this type of malocclusion poses a real challenge in orthodontic practice [9]. The incidence of twisted roots, malformed crowns and dentigerous cysts is also significantly elevated in CCD patients [20].

1.1.4. Radiographic Findings

Radiography is considered a very important diagnostic tool to confirm the diagnosis of *Cleidocranial Dysplasia*. It reveals the various abnormalities of the craniofacial region, dentition, clavicles, and pelvis. Radiographs of the skull classically exhibit patent fontanels and wormian bones, broad and anomalous cranial sutures, in addition to underdeveloped paranasal sinuses. The clavicles may be hypoplastic, unilaterally or bilaterally appearing as small fragments attached to the sternum process, or may be completely absent (aplastic). The mandible and maxilla contain many unerupted and supernumerary teeth which are often malpositioned and distributed in different areas of the jaws [24]. Such dental findings can be easy distinguished by orthopantomogram (OPG) *(Fig.4)*.

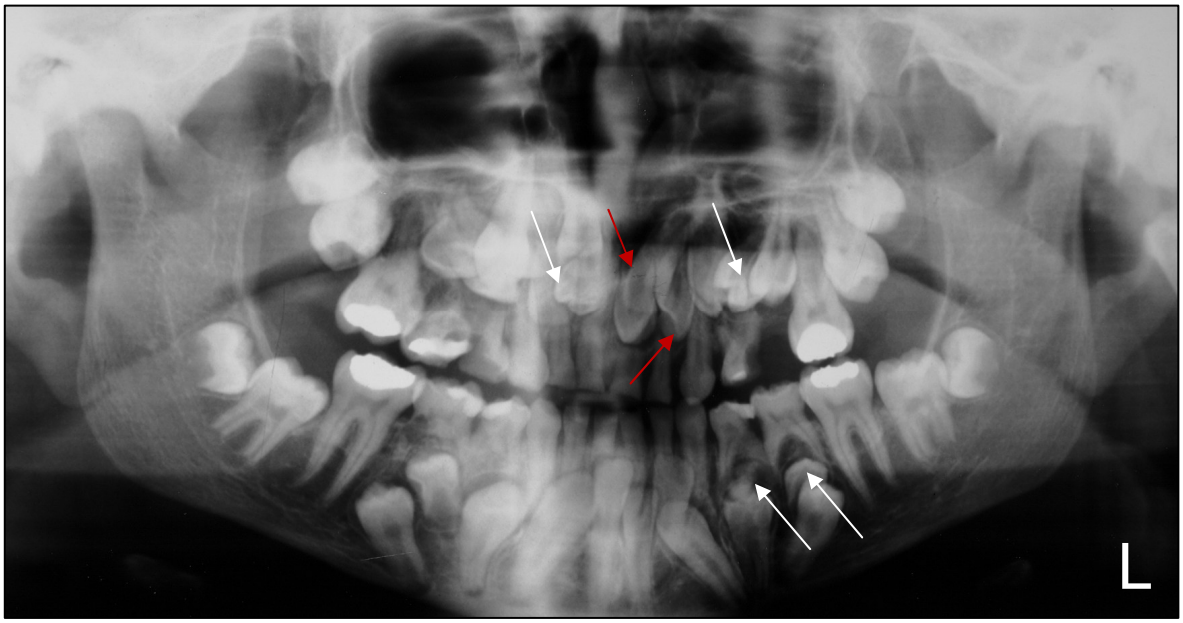


Fig.4. OPG of 15,4 years old patient, who was admitted in the orthodontic clinic in Regensburg University Hospital, due to delayed eruption of the permanent teeth, multiple retained teeth and presence of supernumerary teeth. January 2001. Note the supernumerary teeth marked with white arrows and the severely rotated and malpositioned permanent teeth marked with red arrows. *Regensburg University Hospital, Orthodontic Clinic Archive*

1.1.5. Treatment

There is no definite treatment protocol for patients with *Cleidocranial Dysplasia*. Genetic counseling is the first step in decision making and treatment planning. Early diagnosis of the syndrome is very critical for correct timing of treatment onset, especially regarding the various dental and maxillofacial anomalies [26, 27]. Ideally, therefore, the diagnosis should be made before the age of 9 [17].

The present mode of treatment of the dental anomalies combines both early surgical procedures and orthodontic therapy, in addition to some protective procedures. In the early childhood, wearing a protective headgear is highly recommended; especially while the fontanels remain patent.

As a first step in the combined dental treatment, all supernumerary teeth and over-retained primary teeth should be surgically extracted, when the root formation of succedaneous teeth exceeds 50%. This is followed by surgical exposure of unerupted teeth. Many researchers reported that early surgical exposure of unerupted teeth may result in a stimulation of cementum formation and spontaneous eruption of the dentition with normal physiologic root formation. However, in most cases, the permanent teeth fail to erupt physiologically, which requires orthodontic intervention to move the teeth to the level of occlusion using special appliances with controlled orthodontic forces.

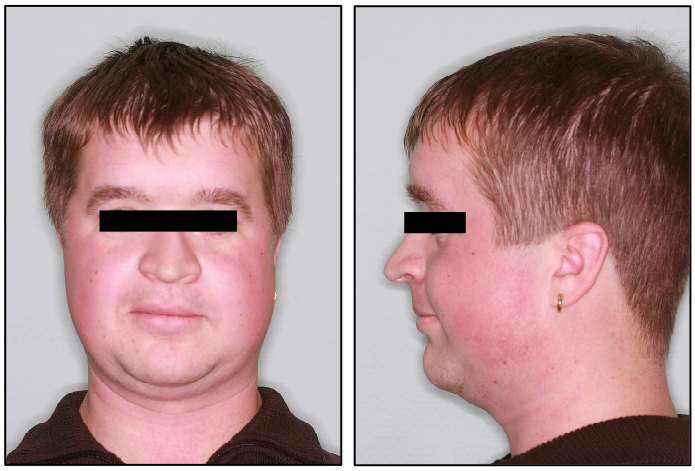


Fig.5. Extra-oral photos of the previously demonstrated patient (23 years old) with CCD syndrome. The photos show considerable improvement in his profile and facial esthetics after he had been operated in the Maxillofacial Surgery clinic in Regensburg University Hospital. Note the surgical reposition of both jaws. *Regensburg University Hospital, Orthodontic Clinic Archive*

Due to complete failure of tooth eruption, even when orthodontically assisted, orthognathic surgery, postsurgical orthodontic therapy, and prosthetics are anticipated in many cases with dentofacial disharmony [9] *(Fig.5)*.

1.2. RUNX2

The *Runx2* gene (osteoblast-specific transcription factor) [28], also known as Core-binding factor α1 (CBFα1), PEBP2A1, and AML3, is considered to be the master transcription factor of bone and plays a major role in all phases of bone formation. It is also responsible for the initial turnover of pluripotent mesenchymal cells into osteoblasts [29].In addition, its presence controls the proliferation, differentiation, and maintenance processes of these cells [16, 30, 31]. Its involvement in the development of calcified tooth tissue, and its influence on proliferation of the dental lamina, makes *Runx2* a very important factor also in the later stages of tooth formation [31-33]. Furthermore, *Runx2* regulates the alveolar remodeling process, which is essential for tooth eruption and may play a role in the maintenance of the periodontal ligament integrity [29].

Mutations or deletions of *Runx2* have been identified as the responsible and causative factor of the Cleidocranial Dysplasia syndrome [22]. This has been proved by a large number of in vitro experiments and finally confirmed by the complete absence of ossification in *Runx2*-knockout mice [30, 34-36].

This gene belongs to the runt domain (RUNX) family of genes, which consists of three members: Runx1, *Runx2* and Runx3.These genes have been identified and localized on human chromosomes 21q22.12, 6p21 [22] and 1p36.1 respectively [36-40].

1.2.1. Runx2 Structure and Expression

The *Runx2* gene spans approximately 220 kb of the 6p21 human chromosome, contains eight exons [16, 41, 42] and shares significant structural similarity with other Runx-family members. The runt domain has an s-type immunoglobulin structure and mediates binding to the core DNA sequence (5'-PuACCPuCA-3') and its complement (5'-TGPyGGTPy-3') [39, 40].

The expression of the *Runx2* gene is initiated by two promoters separated by exon 1 and a large intron. The distal and the proximal promoter (P1 and P2, respectively) generate two major transcripts (proteins) isoforms, namely type II (starting with the sequence MASNS) and type I (starting with the sequence MRIPV), respectively *(Fig.6)*. It is well-known that both protein isoforms are expressed in osteoblasts and in terminal hypertrophic chondrocytes, however,

type I isoform expression has also been detected in many other non-osseous tissues [43].

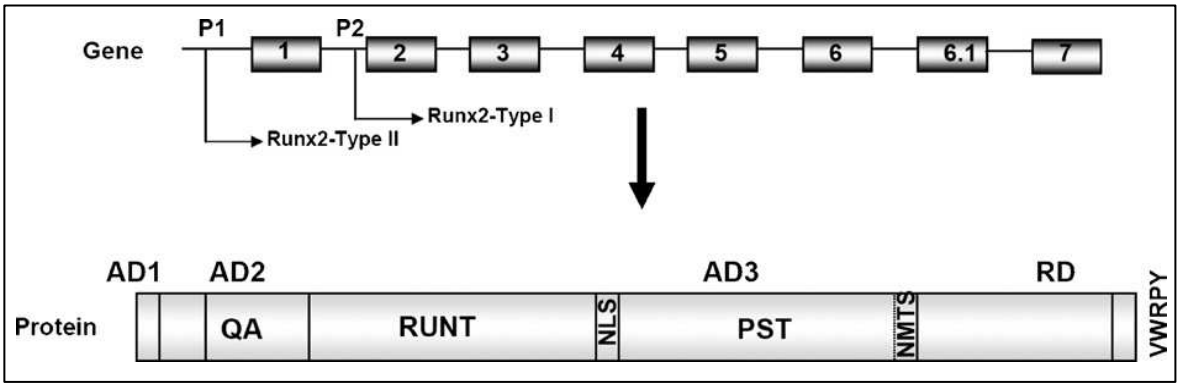


Fig.6. Schematic illustration of the Runx2 structure. The *Runx2* gene comprises 8 exons and its transcription is controlled by two promoters, P1 and P2, which give rise to transcripts/protein isoforms, type II and type I, respectively. The Runx2 proteins contain multiple functional domains: AD1-3, transactivation domains; QA, glutamine/alanine rich domain; RUNT, runt homology domain; NLS, nuclear localization signal; PST, proline/serine/threonine rich domain; NMTS, nuclear matrix targeting signal; RD, repression domain; VWRPY, conserved repression "domain" of runt proteins [44]

Runx2 binds to DNA interacting with the small protein core binding factor (CBF), which is a common component of all Runx proteins. In normal instances, CBF does not bind to DNA itself; however, it has the ability to enhance the DNA-binding capacity of Runx proteins and to increase their half-life stabilizing them against proteolytic degradation by the ubiquitin–proteasome system [45].

The transcriptional activity of *Runx2* is mediated by multiple activation and repression domains. *Runx2* contains three activation domains: AD1, AD2 & AD3. The domain (AD3), which is the principal activation domain, is a proline/serine/threonine (PST)-rich region positioned near the C-terminal part of *Runx2*. However, both domains AD1 and AD2 are located at the N-terminal. AD2 is located in a region adjacent to the N-terminus of the protein which is rich in glutamines and alanines (QA domain). The latter two domains have the ability to potentiate the maximal transcriptional capacity of *Runx2*. The last five C-terminal amino acids, known as VWRPY motif, are a common characteristic of all Runx proteins and show a repression activity *(Fig.6)* [46].

Like any other members of the Runx family, *Runx2* is able to repress transcription independently of TLE/Groucho proteins, through interaction with other co-repressors such as histone deacetylases [46]. The localization of *Runx2* is regulated by a 9-amino acid sequence positioned at the junction of the runt and

PST domains. This nuclear localization signal (NLS) is rich in basic residues and can be seen in other runt-related proteins *(Fig.6)* [47]. Besides the nuclear localization of *Runx2*, it exhibits a subnuclear topography as well, which is required for maximal transcriptional activity. A 31-amino acid Nuclear Matrix Targeting Signal (NMTS) directs *Runx2* to subnuclear foci and reinforces its integration with the nuclear matrix [48].

1.2.2. Biological Functions of Runx2

Osteoblasts are the key bone cells responsible for building and repairing bone. It is well-known that *Runx2* has a critical role in osteoblast differentiation, function and, consequently, in bone biology. *Runx2* determines, at the early stage of embryogenesis, the osteoblast lineage from multipotent mesenchymal stem cells, but inhibits this process at the late stage [31].

Runx2 binds to OSE2 sites which are found in the promoter region of all major osteoblast related genes, thus controlling their expression. Such ectopic expression of *Runx2* in mesenchymal cell lines leads to up-regulation of these osteoblast-related genes like osteocalcin, alkaline phosphatase, collagenase-3, bone sialoprotein and collagen type I [30]. Several studies have shown that in homozygous mice with a mutated *Runx2* gene, there is a complete lack of ossification and both intramembranous and endochondral ossification are totally blocked due to the maturational arrest of osteoblasts [35]. As a result, *Runx2*-knockout mice display an absence of osteoblasts and bone formation, while the heterozygous mice show specific skeletal abnormalities that are very similar to and characteristic of the human heritable skeletal disorder, Cleidocranial Dysplasia (CCD) [36] *(Fig.7)*.

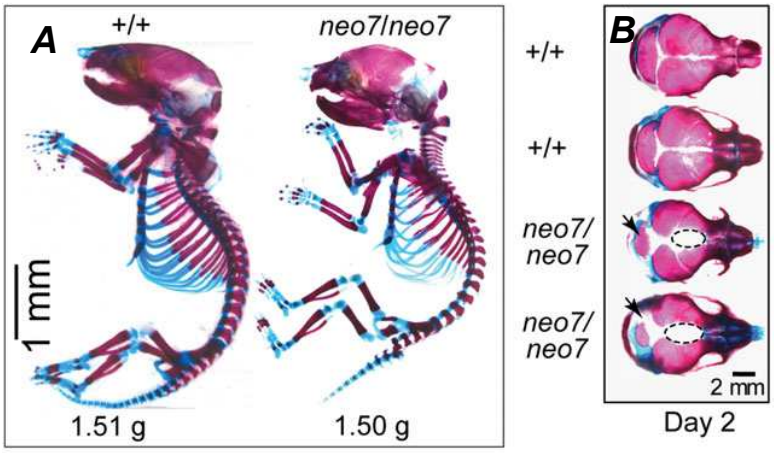


Fig.7. Runx2 homozygous mice display defects of the cranium and clavicles during post-natal growth. The skeletal phenotypes were determined using alizarin red/alcian blue (AR/AB) stains for bone and cartilages respectively. *(A)* The Hypoplastic clavicles. *(B)* The skull shows cranial defects including a wide suture, a decreased basis sphenoid bone (arrow) and non-osseous tissue present in the junction of the posterior frontal suture and the coronal suture [(49)]

Runx2 is also expressed in hypertrophic chondrocytes and is involved in their differentiation process [(50, 51)]. Despite the crucial importance of *Runx2* proteins for bone formation and repair, tissue-specific over-expression of the same proteins in transgenic mice leads to osteopenia, decreased bone mineral density and multiple fractures and bony defects [(52)]. These findings have demonstrated the importance of regulation of *Runx2* expression and its activity in bone pathology and biology.

Regarding the different cellular functions of the two major *Runx2* isoforms (type I and type II), a number of studies suggest that both isoforms are engaged in the stimulatory action of osteoblast differentiation. However, they support separate functions at different stages of osteoblast differentiation and bone formation. The two affected processes of bone formation are the endochondral and intramembranous stages [(43)]. It has been proved that the type I isoform is mainly involved in intramembranous bone formation, whilst the type II isoform has an exclusive role in endochondral bone formation [(53)]. Type I isoform is widely expressed in osteoprogenitor cells and in active osteoblasts during Intramembranous bone formation, whereas type II isoform expression is only seen in cells lining mineralized bones [(43, 53)].

Runx2 is responsible for mediating and coordinating multiple signaling pathways that affect osteoblast function. A variety of chemical and physical stimuli including those initiated by ECM, osteogenic growth factors (e.g. bone morphogenetic proteins (BMPs) and fibroblast growth factor-2 (FGF-2)), hormones (e.g. parathyroid hormone and growth hormone) and mechanical loading (strain/stretching) can modulate the phosphorylation/activation status of *Runx2*. Moreover, they can support its interaction with other proteins, such as the transcriptional activators (e.g. activator protein-1 (AP-1) and SMADs), transcriptional repressors and co-repressors, histone acetylases and deacetylases [54-56].

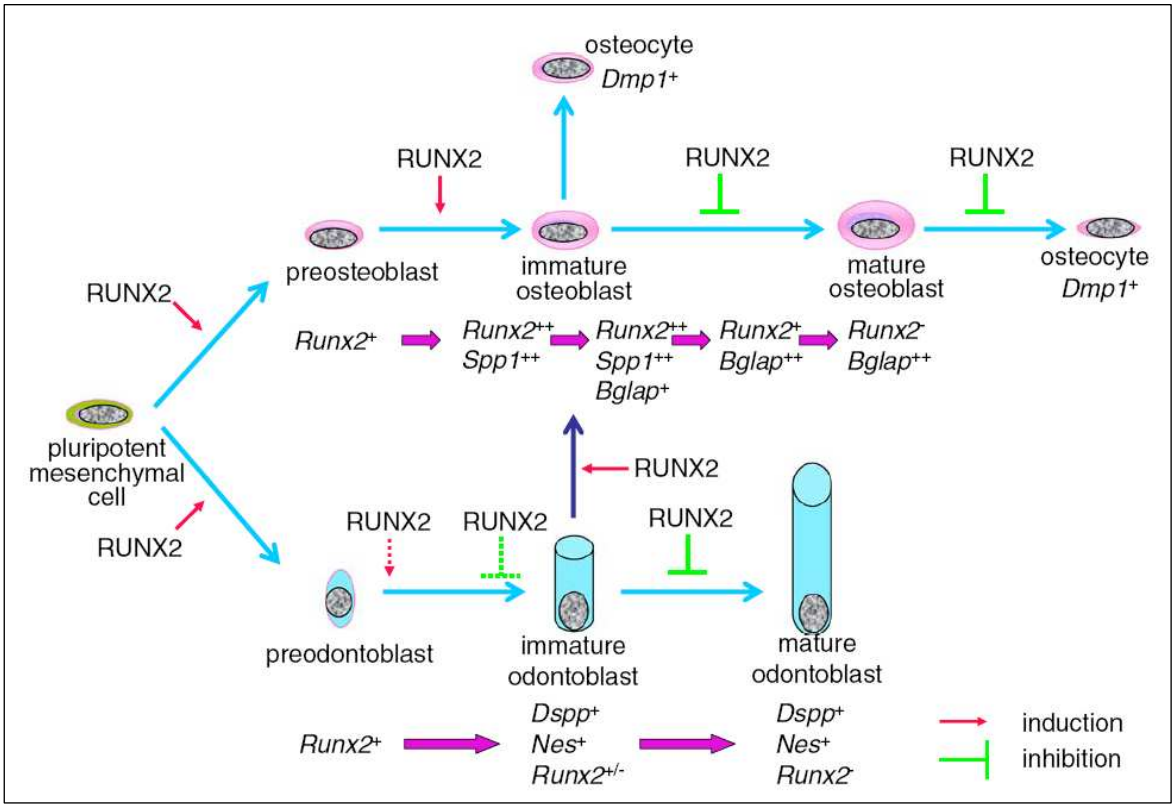


Fig.8. This diagram demonstrates the regulating effect of *Runx2* on osteoblasts and odontoblasts differentiation. *Runx2* promotes the differentiation of pluripotent mesenchymal cells into osteoblasts and odontoblasts lineage, increases the number of immature cells, but inhibits their maturation. It has been noticed that preosteoblasts and preodontoblasts express Runx2 abundantly at the early stages of cells differentiation. Later, however, it will be down-regulated by immature osteoblasts and odontoblasts. In mature cells Runx2 has an inhibiting effect of the terminal differentiation, and therefore, is not expressed anymore by these cells [57]

1.2.3. Runx2 and Bone Tissue

Bone tissue mainly consists of hydroxyapatite crystals beside various types of other extracellular matrix (ECM) proteins, including type I collagen, osteocalcin, osteopontin, osteonectin, bone sialoprotein (BSP), and proteoglycans [58, 59]. These bone matrix proteins are secreted and deposited via polarized mature osteoblasts aligned on the bone surface [60]. Many studies were focused on the precise role of matrix proteins in the formation of bone. Until now, however, their actions still are not fully explained [61, 62]. Osteoblasts also regulate the formation of hydroxyapatite crystals.

The experiments of *Runx2*-null mice clearly demonstrated that this transcription factor is critical for osteoblast differentiation, as these mice showed no bone tissue, osteoblasts or osteoclasts, despite normal cartilaginous skeletal patterning. Chondrocyte maturation, however, is disturbed in consequence [35, 36].

Runx2 expression is also very critical for mature osteoblasts. Mature mice, in which active *Runx2* levels have been reduced, exhibited a decreased expression of the genes encoding the main bone matrix proteins, such as osteocalcin, osteopontin, BSP and collagen type I [31]. These genes are regulated through the *Runx2*-binding sites in the proximal promoter segments of the mentioned genes [63]. Thus, it has been proved that *Runx2* is essential for normal bone formation, while insufficient levels regardless of their reason result in disturbed bone formation.

Over-production of *Runx2* proteins will also affect bone formation. Osteoblasts taken from non-syndromic synostosed sutures in children showed an increase in *Runx2* expression, which explains the enhanced proliferation and bone-forming ability of these cells [64]. Many researches demonstrated that *Runx2* expression decreases with age which could be a possible explanation for impaired osteoblast function and reduced bone formation with aging [65].

Runx2 expression is affected by a diversity of signaling pathways. The *Runx2* gene, in fact, plays a central role in coordinating multiple signaling pathways affecting osteoblast differentiation [66] *(Fig.8)*.

1.2.4. RUNX2 Domains

Runx2 binds to the core binding factor site, which is also known as the osteoblast-specific cis-acting element 2 (OSE2) [67]. The RHD is responsible for the DNA-binding properties of *Runx2*.

One major repression domain and three transactivation domains have been recognized in the *Runx2* protein [47]. We will start here explaining the various activation domains of *Runx2* gene.

The first transactivation domain is located in the N-terminal 19 amino acids of the protein, while the second is located in the glutamine/alanine (Q/A) domain. The transactivation of Q/A domain depends on a stretch of 29 glutamine residues. Interestingly, deletion of the alanine stretch does not affect the transactivation process; however, expansion has a repressive effect [22]. In addition, this expansion may also play an essential role in nuclear localization [68]. The third transactivation domain is present in the N-terminal portion of the proline/serine/threonine (PST)-rich domain. A mutation in this region has been shown to cause a failure to interact with SMADs, which may accordingly reduce the response of osteoblasts to the Transforming growth factor-b/Bone morphogenetic protein (TGF-b/BMP) signaling pathway [69]. This region has also been shown to interact with the co-activator molecule (p300), affecting in result the expression of the osteocalcin gene [70]. This action is independent of the acetyl-transferase activity of p300, which also protects *Runx2* from degradation by SMAD ubiquitin regulatory factor (SMURF)-mediated ubiquitination and also increases the transactivation potential of *Runx2* [71].

The C-terminal part of the PST domain is a repression domain [47, 72]. The terminal five amino acids (the VWRPY motif) are very highly conserved and have the ability to bind to the co-repressor proteins of the transducin-like enhancer of split (TLE) or Groucho related genes (Grg) family [73].

The down-regulation and repressing of TLE/Grg expression during osteoblast differentiation is considered a very essential mechanism for relief of *Runx2* repression during cell differentiation [74].

Other parts of the *Runx2* gene have been shown to react with other co-repressors, such as histone deacetylases (HDAC) [72], SIN3 [75], and yes-associated proteins (YAP) [76]. After the transcription process, *Runx2* will be imported to the nucleus

where it binds to specific regions of the nuclear matrix to effect transcriptional control [77], co-localizing with co-activators (e.g. SMADs [78] and RNA polymerases) at nuclear sites that support RNA synthesis [48].

This function is affected by the nuclear matrix targeting signal (NMTS) region, which is a 38 amino acid segment situated between the RHD and PST domains [79]. Studies have demonstrated that point mutations in the NMTS region directly affect the intranuclear localization of *Runx2* which in turn affects its interaction with target genes involved in osteolytic activity [69, 80]. The interaction of transcription factors with cellular signal transducers at particular points in the nuclear matrix may somehow explain the tissue-specific action of the *Runx2* proteins and of transcription factors in general.

1.3. Strontium Ranelate

In the last decade, several effective medical compounds have been introduced for treatment of osteoporosis. Vertebral and non-vertebral fractures were decreased by 30-65 % through treatment with anti-catabolic agents (which decrease the bone resorption rate) or anabolic agents (which increase the bone formation and mineralization rate) [81].

1.3.1. Chemical Structure and Characteristics

Strontium ranelate (SR) is a new chemical compound composed of two atoms of stable Strontium (Sr^{2+}) and an organic acid (ranelic acid), which acts as a carrier [82, 83] *(Fig.9)*.

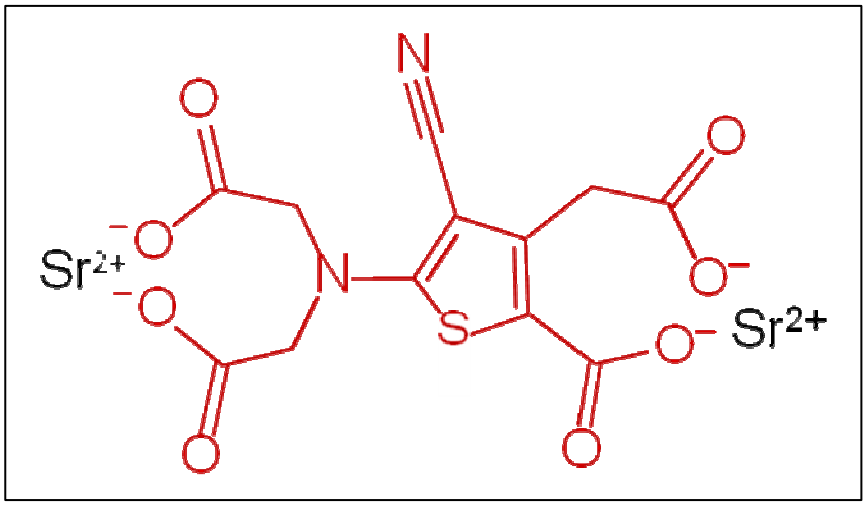


Fig.9. Strontium Ranelate, systematic (IUPAC) name: distrontium 5-[bis (2-oxido-2-oxoethyl) amino]-4-cyano-3-(2-oxido-2 oxoethyl) thiophene-2-carboxylate; chemical formula: $C_{12}H_6N_2O_8SSr_2$. The figure demonstrates the chemical structure of SR. The molecule of the organic ranelic acid is marked with red color, while both Strontium atoms were marked with black color. [84]

The mineral Strontianite is named after the Scottish village of Strontian which has been discovered in the lead mines there in 1787 [84]. However, Strontium itself was discovered in 1798 by *Thomas Charles Hope* [85]. It occupies a middle position between Calcium (Ca^{2+}) and Barium (Ba) in the periodic table with atomic number 38. Many studies have demonstrated the high atomic and ionic similarity between Calcium and Strontium [86].

Strontium ranelate represents a new generation of oral anti-osteoporotic agents electively concentrated in positions of active bone formation and exerting both anti-catabolic and anabolic effects on bone cells [81]. It was first used in the treatment of osteoporosis in the 1950s. Strontium itself is not found in its free

form, because of its high tendency to oxidate spontaneously even at room temperature turning to yellow color as a result of oxide formation. It is found in different concentrations in some natural sources such as vegetables and cereals [87].

1.3.2. Medical and Biological Functions

Strontium ranelate has the ability to reduce the risk of vertebral [88], non-vertebral and hip [89, 90] fractures in postmenopausal osteoporotic women (PMOP).

SR increases bone formation and decreases bone resorption rates [90-97] which results in the prevention of bone loss and a general increase in bone mass and strength [83, 95, 98-101]. This unique effect on the bone remodeling process makes Strontium ranelate the only anti-osteoporotic agent, which can rebalance the bone turnover process in favor of bone formation. It is therefore referred to as a "dual action bone agent" (DABA) [88].

Many animals and in-vitro experiments [94] as well as measurements of biochemical markers of bone turnover in clinical trials on osteoporotic women [88] have demonstrated the promoting effect of Strontium ranelate on bone formation and depressing effect on the bone resorption process. Several experiments on ovariectomized (OVX) or immobilized osteopenia rats have confirmed that Strontium ranelate could totally prevent bone loss and resorption in the experimented animals [102-104].

At the macroscopic level, recent studies reported that the increase in bone strength induced by Strontium ranelate in rats may be explained by improvement of the micro architecture as well as the intrinsic bone tissue quality measured by hardness and dissipated energy upon nano-indentation [99]. The anabolic effect of strontium on bone metabolism have been also observed and reported in intact animals, such as mice [105], rats [98] and monkeys [105].

Microscopically, Strontium has been shown to enhance and significantly promote the replication and the functional activities on bone surfaces. It is able to increase osteoblast replication, osteoblast differentiation, collagen type I synthesis and bone matrix synthesis and mineralization [92, 93, 96, 106].

On other hand, it inhibits the formation of osteoclasts and decreases the number of differentiated osteoclasts [92]. Further investigations have reported an induction of apoptosis of mature osteoclasts [107-109].

Moreover, analysis of bone markers detected that strontium ranelate reduces the urinary excretion of the bone resorption marker, type I collagen N-telopeptide, while it increases the levels of a serum marker of osteoblast differentiation and bone-specific alkaline phosphatase [110].

Thus, the functions of Sr^{2+} can be summarized as follow:

1. Reducing the incidence of vertebral, non-vertebral and hip bone fractures in PMOP.
2. Promoting the bone remodeling process by increasing bone formation and decreasing bone resorption rates.
3. Increasing bone strength and mass and improving bone architecture quality.
4. Supporting the differentiation of MSCs into osteoblasts.
5. Accelerating the maturation of osteoblasts.
6. Increasing the synthesis of collagen type I and mineralization of bone matrix.
7. Suppressing the formation and function of osteoclasts and promoting osteoclasts apoptosis.
8. Controlling urinary excretion of the different bone markers in favor of decreasing bone resorption and increasing osteoblast differentiation and bone formation and deposition.

1.3.3. Signaling Pathways

Despite the huge number of studies and experiments conducted on Strontium ranelate and its mode of action, the precise molecular mechanisms involved are still elusive and unclear. However, there is strong evidence that the calcium-sensing receptor (CaSR), which is a critical factor for bone development and turnover processes [111], is involved in the actions of Strontium ranelate [112-118]. Because of the atomic and ionic similarity between Strontium (Sr^{2+}) and Calcium (Ca^{2+}), it has been hypothesized that Sr^{2+} may also act as an agonist of the extracellular CaSr expressed in all stages of osteoblasts differentiation and maturation [119, 120].

Studies have shown that within physiologic Ca^{2+} concentrations, Strontium ranelate can induce further CaSR activation comparing to Ca^{2+}. It has also been demonstrated that Strontium ranelate is less potent than Ca^{2+} in stimulating inositol phosphate accumulation, but almost equals Ca^{2+} in stimulating extra-cellular signal-regulated kinase (ERK) phosphorylation and a non-selective cation channel which strongly suggest that both Ca^{2+} and Strontium ranelate have differential cellular effects [121]. Moreover, the retardation in activation of mitogen-activated protein (MAP) kinase and protein kinase C (PKC) pathways mediated by Strontium ranelate compares to activation mediated by $CaCl_2$. Thus, the existence of molecular mechanisms at least partially separate from Ca^{2+} [122] is supported.

One of the recent studies on rat primary osteoblasts confirmed that the proliferation and the expression of genes involved in osteoblast proliferation and differentiation, such as (c-Fos) and (Egr-1), was stimulated by Strontium ranelate. Conversely, these effects were suppressed by over-expressing the dominant negative CaSR which suggests that Sr^{2+} action was mediated by Calcium-sensing receptor mechanisms [121]. Similar results were obtained in other studies, where the Strontium ranelate-induced stimulation of replication was completely inhibited by transfection of a dominant negative CaSr [121,123].

As a conclusion, Strontium ranelate can be considered as a full CaSr agonist in primary osteoblasts and its proliferative effect on these cells can be, at least partially, explained by a CaSr-dependent mechanism [82].

1.3.4. Differentiation of Mesenchymal Stem Cells into Osteoblasts

Strontium Ranelate promotes the differentiation from early progenitor cells to mature osteoblasts by increasing the expression of osteoblastic markers such as Runt-related transcription factor 2 (Runx 2), alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OC) through Calcium-sensing receptor mechanisms [92]. In vitro, bone marrow stromal cells exposed to a differentiating medium for 21 days and treated with Strontium ranelate showed an increase in the transcriptional activity and phosphorylation level of Runx2 gene, but not of osteocalcin [96]. On other hand, Sr^{2+} treatment of mature osteoblasts induced only minimal effect on Runx2 expression, but had a positive effect on osteocalcin. This can be explained by the critical role of Runx2 in promoting and initiating the MSCs differentiation to osteoblasts.

However, osteocalcin (OC) is a known negative regulator of the differentiation process, which is normally expressed in the late stages [96, 124].

In addition to the promoted osteogenic differentiation, the increased phosphorylation of Mitogen-activated Protein Kinase (MAPK) ERK1/2 and p38 was also detected in Strontium-treated MSCs [126].

MAPK has a direct role in enhancing osteogenesis through the phosphorylation and subsequent activation of Runx2 [127].

It has also been demonstrated, that Rat Sarcoma viral oncogene homolog (RAS), an upstream regulator of ERK1/2 and p38, was activated by Strontium treatment, and siRNA-mediated RAS knockdown inhibited Strontium-stimulated expression of osteogenic markers. Thus, Strontium could also stimulate the osteogenic differentiation of MSCs through activating the RAS/MAPK signaling pathway and the downstream transcription factor Runx2 [126].

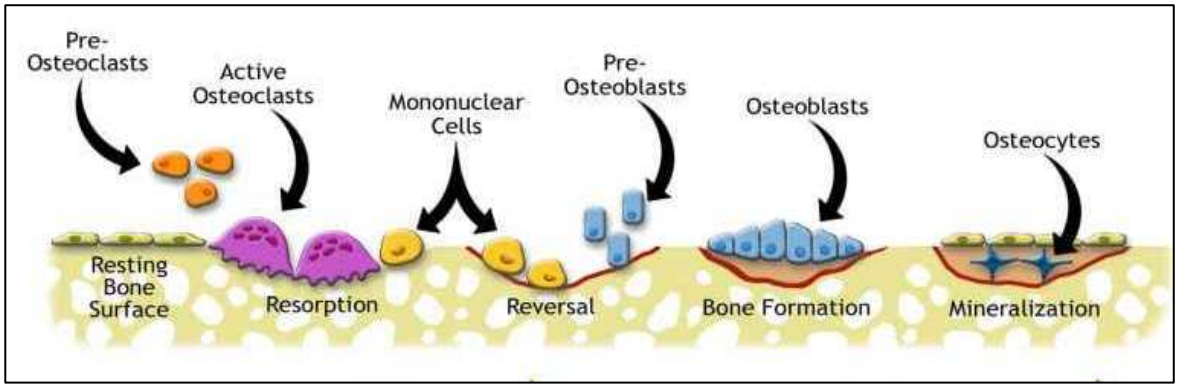


Fig.10. Shows the different types of bone cells with their related main functions. Osteoprogenitor cells are the main source of all bone cells. Osteoclasts are related to bone destruction and resorption, while osteoblasts induce a reversal action and lead to bone formation. Osteocytes are represented an inactive osteoblasts embedded in mineralized bone [125]

1.3.5. Differentiation, Activity and Apoptosis of Osteoclasts

The suppressive effect of Strontium ranelate on osteoclasts is one the critical actions that play a major role in the Sr^{2+} mediated treatment of osteoporosis leading to a drop in bone resorption and the destructive process. S^{2+} has the ability to decrease the differentiation rate, the activity and total number of bone-resorping osteoclasts though its pro-apoptotic effects [107-109].

This effect was recently confirmed in a series of experiments. The number of mature osteoclasts derived from murine spleen cells and their resorbing activity were strongly suppressed after Strontium ranelate exposure in a process directly

associated with the disruption of the actin cytoskeleton of the osteoclast-sealing zone [92].

Moreover, monitoring the expression of carbonic anhydrase and fibronectin receptors after cells treatment with Strontium ranelate demonstrated its destructive action on osteoclasts [91].

Several studies support the idea that the effect of Strontium ranelate is dose-dependent and is exerted, at least partially, through a CaSr-dependent mechanism. The stimulation of Calcium-sensing receptors by Sr^{2+} has been demonstrated to be the underlying major mechanism of the cellular effects of strontium ranelate on these cells [128].

This mechanism is mediated through direct activation of CaSr followed by stimulation of phospholipase C (PLC) and nuclear translocation of NF-κB. However, and in contrast of Ca^{2+}, the effects of Sr^{2+} are mediated by activation of diacylglycerol (DAG)-PKCβII signaling and not by IP3-dependent signaling [80] *(Fig.11)*.

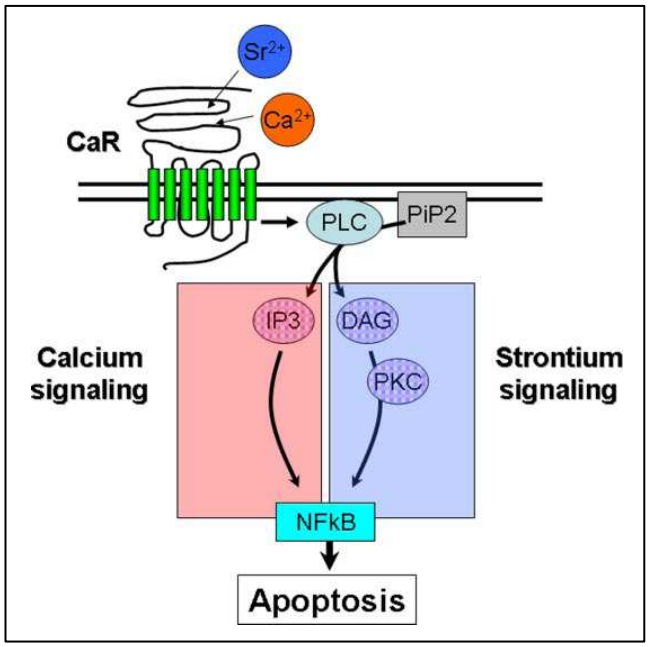


Fig.11. This diagram demonstrating the role of CaSR in both Ca^{2+} and Sr^{2+} induced apoptosis of mature osteoclasts:
After stimulation by extracellular calcium, the CaSR activates PLC, which is responsible for the translocation of NF-κB from the cytoplasm to the nucleus in mature osteoclasts, in an IP3-dependent manner.
However, stimulation by extracellular strontium, the CaSR also activates PLC, which is, in turn, responsible for activation of the DAG-PKCβII signaling pathway, which in turn promotes translocation of NF-κB from the cytoplasm to the nucleus in mature osteoclasts but in an IP3-independent manner. Activation of NF-κB can potentiate, leading to enhanced apoptosis of mature osteoclasts [80]

2. PROBLEM STATEMENT

Insufficient or completely impaired production and expression of the *Runx2* transcription factor is the leading factor of the Cleidocranial Dysplasia syndrome. Such a mutation causes a deficiency or inhibition of osteoblast differentiation from mesenchymal stem cells and subsequent defects and deformities in both bone and tooth formation.

Cleidocranial Dysplasia is frequently associated with various symptoms and characteristic features ranging from mild, in some cases not noticeable, symptoms to severe and challenging symptoms. Many patients are facing real difficulties in their daily life as a result of the skeletal deformations and possible other associated abnormalities such as muscular hypotonia and hearing loss. In addition, these patients suffer from esthetic problems due to the presence of supernumerary teeth, delayed eruption of permanent teeth, associated malocclusion and the hypoplastic maxilla responsible for the prognathic appearance of these patients.

Treatment and management of the CCD symptoms is still a real challenge and subject of continuous research. Numerous studies aimed to find the best management methods of the associated symptoms some of which, however, still are untreatable and unmanageable.

In view of the increasing number of affected patients worldwide and our experience with such cases at the Orthodontic Clinic of Regensburg University Hospital, new approaches to the management of the associated symptoms and their severity had to be considered.

3. AIM OF THE STUDY

It is well-known that patients with Cleidocranial Dysplasia suffer from a mutation of the Runx2 gene which results in impaired bone formation and remodeling processes. Because of the high potential of Strontium in promoting the expression of Runx2 and other osteoblast-related genes and stimulating the differentiation of the mesenchymal stem cells into osteoblasts, we decided to apply this chemical compound to cells extracted from a patient with Cleidocranial Dysplasia syndrome.

Due to its ability to improve osteoblast differentiation and, hence, bone formation, .Strontium is recognized and registered in the European Union (EU) for treatment of women with osteoporosis. Moreover, it shows a high potential to decrease bone resorption due to its depressive role in osteoclast formation and its promoting effect in osteoclast apoptosis. This dual effect has made Strontium the treatment of choice for osteoporosis.

After reviewing an abundance of articles, we failed to find one single study about the use of Strontium in dentistry. Therefore, we aimed to explore the effects of Strontium on osteoblasts extracted from a patient with CCD and to demonstrate possible improvements in the expression of the Runx2 transcription factor or in osteoblast function.

This pioneering treatment approach non only focuses on the management CCD symptoms, but rather promises to prevent the very development of the symptoms or at least to decrease their severity. If this experiment succeeds, further animal and clinical trials in the embryonic stages of development may improve the prospects of eliminating or limiting CCD symptoms and getting healthy kids from affected parents.

4. MATERIALS AND METHODS

4.1. Cell Culture

4.1.1. Isolation of the Osteoblasts

Primary Runx2 $^{+/-}$ osteoblasts were obtained from human alveolar bone following appropriate informed consent of a patient with Cleidocranial Dysplasia. The analysis of genomic DNA of the patient revealed a mutation in the runt-domain of the Runx2 gene and confirmed his/her affection with the CCD syndrome. The patient also exhibits characteristic symptoms of CCD such as supernumerary teeth, brachycephaly, and brachydactyly.

4.1.2. Growth Conditions

The primary cell line was enchriched and cultivated in osteoblast growth medium (Provitro/Germany), which was supplied with 10% heat-inactivated fetal bovine serum and 50 µg/ml ascorbic acid, at 37^{o}C with 5% CO_2. The cells were then obtained in the third passage and were cryopreserved and stored in liquid nitrogen until use.

In order to compare the effect of Strontium on Runx2$^{+/-}$ osteoblasts, we cultivated these cells in absence or in presence of 3 mM Strontium chloride $SrCl_2$ (Merck/Germany) for 10 days in cell culture medium.

4.1.3. Cells Proliferation

Osteoblasts were seeded in 96-well plate at a concentration of 2,800 cells per well in 100 µl cell culture medium. In order to observe the proliferative effect of Strontium, we added $SrCl_2$ up to a concentration of 3 mM to the cells (tested group) or we excluded Sr^{2+} from the cells culture medium (control group). 10 µl WST-1 (Roche/Switzwerland) was added per well to both of test and control group and the cells were incubated at 37^{o}C with 5% CO_2 for a maximum time of 3 hours. The increasing absorbance (A=450 nm) of the sample corresponds to the increasing cell number. Absorbance was determined in 1 hour interval from 0 to 4 hours using a microplate reader (Mod. GENios, Fa. TECAN/Austria).

Counting the number of the cells and confirming their concentration and density was done using a *Hemocytometer (Fig.12)*: It consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines and the depth of the chamber are known. Thus, it is possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.

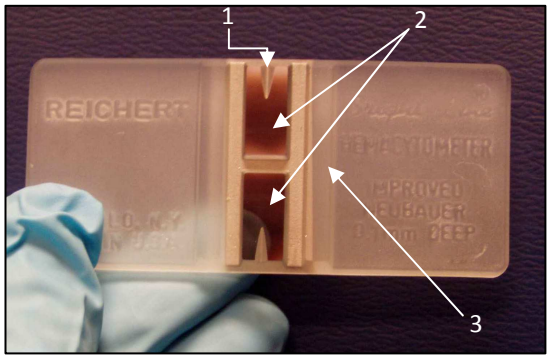
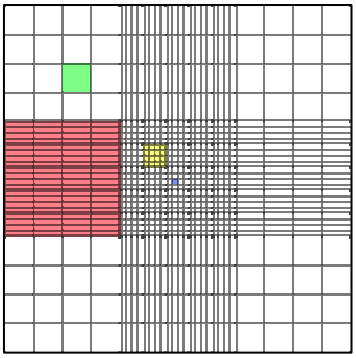


Fig.12. A hemocytometer with the main parts: 1. Sample introduction point, 2. Counting chambers, 3. Cover glass [129]

The concentration or density of the osteoblasts was calculated using this formula:

$$\left(\frac{\text{Number of cells counted}}{(\text{Proportion of chamber counted})(\text{Volume of chamber})}\right)\left(\frac{\text{Volume of sample dilution}}{\text{Volume of original mixture is sample}}\right)$$



Dimensions	Area	Volume at 0.1 mm depth
1 x 1 mm	1 mm^2	100 nl
0.25 x 0.25 mm	0.0625 mm^2	6.25 nl
0.20 x 0.20 mm	**0.04 mm^2**	**4 nl**
0.05 x 0.05 mm	0.0025 mm^2	0.25 nl

Fig.13. Diagram of the hemocytometer grid used to count the number of the tested cells. Note that the different colors represent different dimensions and depths as showed in the adjacent ***TABLE 2*** [129]

4.1.4. Alkaline Phosphatase (ALP) Activity Measurement

This biomedical assay was used for measuring alkaline phosphatase ALP activity is osteoblasts culture samples after been exposed to 3 mM Sr^{2+}. The commercially available cytochemical kit (QuantiChrom™ Alkaline Phosphatase Assay Kit [DALP-250], BioAssay Systems-USA) has been used, in which ALP at alkaline pH catalyses the following reaction:

$$\underset{\text{(Colorless in Alkali)}}{\rho\text{-nitrophenyl phosphate} + H_2O} \xrightarrow{\text{Catalyzing effect of ALP}} \underset{\text{(Yellow in Alkali)}}{\rho\text{-nitrophenyl} + P_i}$$

Confluent adherent cells were trypsinised and counted with a Neubauer-Chamber. Then, 500,000 cells of the test group (3 mM Sr^{2+}) or control group (0 mM Sr^{2+}) were gently lysed in 100 µl CelLytic ™M Cell Lysis Reagent (Sigma/USA) for 20 minutes at 25^0C. Cell debris was removed by centrifugation (13,500g, 4^0C, and 15 min.) of the cell lysat. The volume activity of the alkaline phosphatase was analyzed using a colorimetric assay and normalized to cell number (Sabokbar et al. 1994).

Calculation of ALP activity of the sample (IU/L= nmol/(ml*min)) was done using the following formulas:

$$= \frac{(OD_{Sample\ t} - OD_{Sample\ 0}) * 1000 * \text{Reaction Vol.}}{t * \varepsilon * l * \text{Sample Vol.}}$$

OR

$$= \frac{(OD_{Sample\ t} - OD_{Sample\ 0}) * \text{Reaction Vol.}}{(OD_{Calibrator} - OD_{H_2O}) * \text{Sample Vol.} * t} \times 35.3$$

$OD_{Sample\ t}$ and $OD_{Sample\ 0}$ are OD_{405nm} values of sample at time *t* and 0 min. The factor 1000 converts mmol/L to µmol/L. *t* is the incubation time (min). For *ρ*-nitrophenol, $\varepsilon = 18.75\ mM^{-1} \times cm^{-1}$. *l* (light path, cm) is 1 cm for cuvette, and calculated for 96-well assay from the Calibrator, $l = (OD_{Calibrator} - OD_{H_2O})/(\varepsilon * c)$ [130]

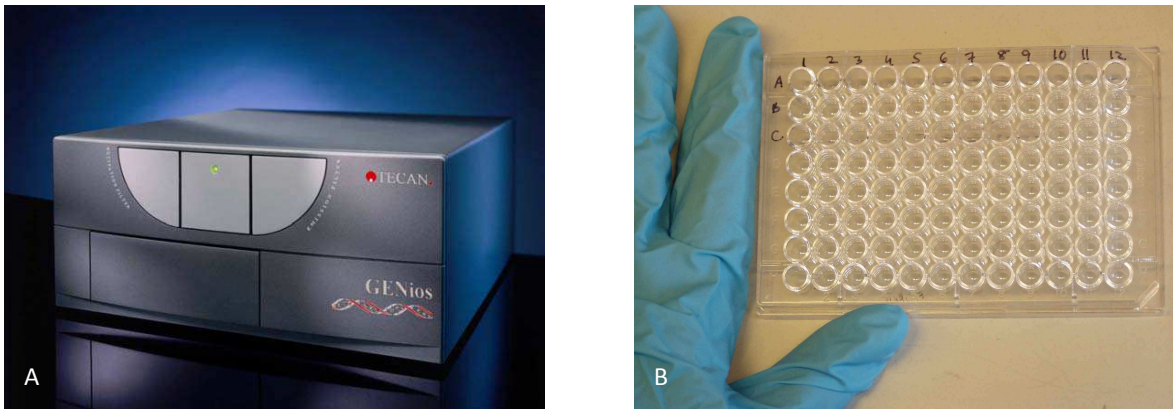


Fig.14.

A. Tecan spectrophotometric plate reader which was used in our study, from GENios, Austria [131]
B. The 96-well microtiter plate used

4.1.5. Biomineralization Assay According to Gregory et.al.

Mineralization of confluent cells was determined by fixation with 10% formaldehyde at room temperature for 15 minutes and subsequent Alizarin red-S staining and quantification as described by Gregory et al. (2004) [132].

4.2. Applied Molecular Biological Methods

4.2.1. RNA Isolation (TRI-reagent)

Adherent confluent cells, which were grown in absence or presence of 3 mM Sr_2Cl for 10 days, were rinsed with 1 ml TRI-Reagent (Sigma/USA) and further processed according to the manufacturer's recommendations to isolate the RNA. The yield of the total RNA was determined using Picodrop™ µl spectrophotometer (Picodrop/UK) at 260 nm. 500 ng of total RNA were applied for cDNA synthesis (QuantiTeck®Reverse Transcriptase, Qiagen/ Germany).

Fig.15. Picodrop™ µl Microliter Spectrophoto-meter for use with Disposable UVpette Tips [133]

4.2.2. cDNA Synthesis

This procedure was performed according to the description in the QuantiTect® Reverse Transcription handbook supplied by QIAGEN ™

1. Template RNA was placed on ice, whereas gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase free water were left at room temperature (15–25°C). All components were centrifuged before use and placed on ice.
2. The genomic DNA elimination reaction was prepared on ice as described in the following table:

TABLE 3. Components of the gDNA elimination reaction according to GIAGEN ™

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer, 7x	2 µl	1x
Template RNA	Variable (up to 1 µg*)	
RNase-free water	Variable	
Total volume	14 µl	–

3. The reaction was incubated for 2 min at 42°C, and then placed on ice immediately.

4. The reverse-transcription master mix was prepared according to the following table:

TABLE 4. Components of the reverse-transcription master mix according to GIAGEN ™

Component	Volume/reaction	Final concentration
Reverse-transcription master mix:		
Quantiscript Reverse Transcriptase	1 µl	
Quantiscript RT Buffer, 5x	4 µl	1x
RT Primer Mix	1 µl	
Template RNA:		
Entire genomic DNA elimination reaction (step 3)	14 µl (add at step 5)	
Total volume	20 l	–

5. Template RNA obtained from step 3 (14 µl) was added to each tube containing reverse transcription master mix.
6. The reaction tubes were incubated for 15 min at 42°C, and then incubated for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. Reverse-transcription reactions were stored on ice and directly preceded real-time PCR.

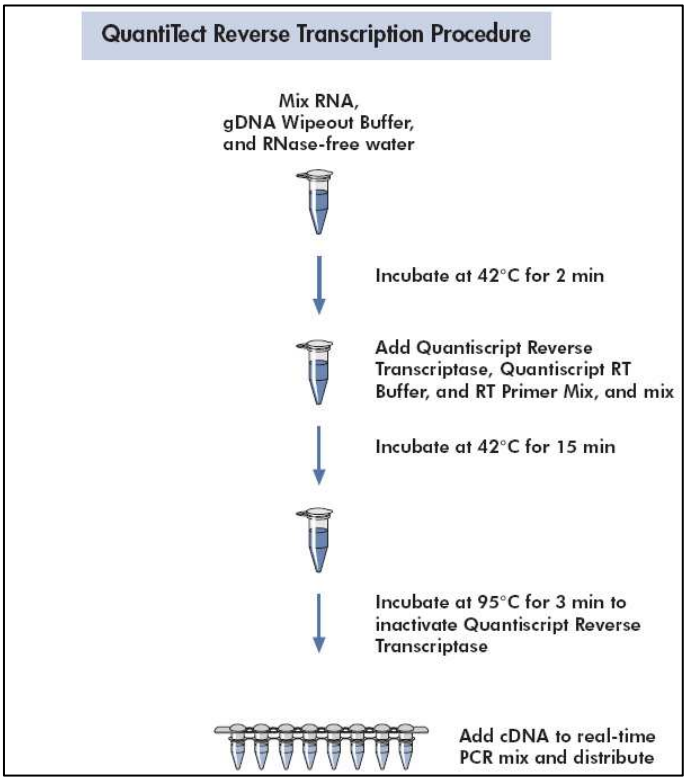


Fig.16. Diagram summarizing the stepwise process of cDNA synthesis from RNA with coincidental elimination of genomic DNA for quantitative real time PCR [(134)]

4.2.3. Real Time PCR

Real time PCR was performed using the DyNAmo™ Sybr Green q-PCR Kit (Finnzymes/Finland) in triplets for each cDNA on Abi7000 (Applied Biosystems/USA). The real time amplifications included an initial step of 10 minutes at 95°C (polemerase heat activation), followed by 40 cycles at 95°C for 10 seconds (denaturation), 65°C for 30 seconds (annealing / elongation / data collection). This technique allows the identification of the cycling point where the PCR product is detectable by means of fluorescence emission (threshold cycle or Ct value). Moreover, a dissociation curve was obtained after each Real time PCR run in order to exclude false-positive signals that might be caused by primer dimmers. Gene expression was calculated using the software tool REST 2005 (Pfaffl et al., 2002). Appropriate primers (Table 5) were chosen by use of the online-program "Primer3" (*http://biotools.umassmed.edu/bioapps/primers_www.cgi)*.

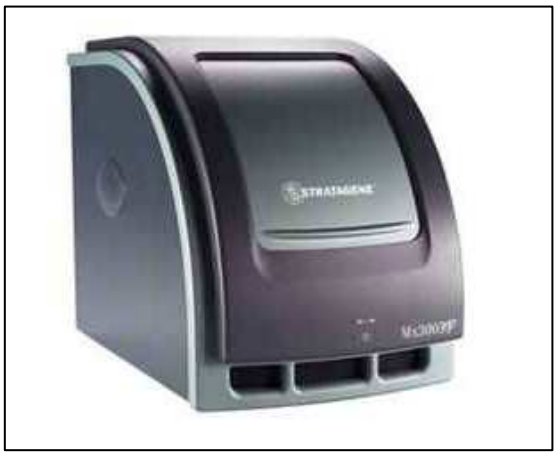


Fig.17. Machine used for q-PCR, from Stratagene-Mx3005®, Germany [135]

TABLE 5. Polymerization chain reaction (PCR) primers used in this study, supplied by Eurofins, Germany. The accession numbers are: NM_167214.1, NM_000478.3 & NM_199173.3 respectively. ALP: Alkaline Phosphatase, OCN: Osteocalcin

eurofins

Name	*Forward*	*Reverse*
18S rRNA	5'-ACGGGGAATCAGGGTTCGAT-3'	5'-TTGCCCTCCAATGGATCCTC-3'
ALP	5'-CCACAAGCCCGTGACAGATG-3'	5'-TTTGGCCCTTGGGGAAGATT-3'
OCN	5'-GGAGGGCAGCGAGGTAGTGA-3'	5'-ACCCTAGACCGGGCCGTAGA-3'

Agarose Gel Electrogenesis: The gel was prepared using 60 ml of 1xTBE (gel buffer) with 120 mg of Biozym LE-Agarose powder (2% agarose gel). The mixture was left to boil at 200°C. After reaching the boiling point and complete dissolving of the agarose, the mixture was removed and left to cool a little at room temperature. Then, 2 µl of ethidium bromide solution (EtBr, 5mg/µl), which intercalates between DNA base pairs and fluoresces when activated with UV radiation, was pipetted into the mixture and mixed by shaking.

10 µl of the sample was taken and mixed with 4 µl of dye buffer with a known migration rate and then placed in the well within the gel. The gel was allowed to set and, then, electrophoresis was carried out with the gel submerged under buffer. The machine was adjusted at 90 V for 60 min. Bands of DNA fluorescence were examined under ultraviolet radiation.

4.3. Statistics

Data analysis was performed using Student's t-test, when appropriate. Otherwise, a Wilcoxon-Mann-Whitney test was employed. Statistical analysis was performed with the SPSS software. Probability values of $p \leq 0.05$ were regarded to be significant.

5. RESULTS

5.1. Gene Expression Analysis of Runx2-Responsive Genes

Various studies noted that Strontium ranelate improves bone quality by increasing bone formation and decreasing bone resorption rates [88-94]. SR acts by means of activation of Runx2 genes and other osteoblastic genes through a Calcium-sensing receptors mechanism [89]. We analyzed, therefore, the gene expression of two Runx2-responsive genes: alkaline phosphatase (ALP) and osteocalcin (OCN), which play a major role in the mineralization process of the bone.

The gene expression of the experimented osteoblastic genes in Strontium-exposed $Runx2^{+/-}$ osteoblasts (test group) was up-regulated *1.3 fold for ALP* and *1.1 fold for OCN*. The following tables show the measurements of gene expression of ALP, OCN and 18S rRNA in both the test and the control group.

TABLE 6. These tables demonstrate the measurements of the tested osteoblastic genes' expression. The left table shows gene expression in 3 mM Sr^{2+} exposed $Runx2^{+/-}$ osteoblasts, whereas the right table shows the control group not exposed to Sr^{2+}. Notice from the last table, the significant up-regulation of ALP and OCN compared to 18S rRNA

Osteoblasts exposed in 3 mM Strontium				
	Alkaline Phosphatase			mean
1	28,67	28,87	28,73	28,756
2	29,55	29,06	29	29,203
3	30,29	29,83	29,71	29,943
4	29,38	29,14	28,85	29,123
	Osteocalcin			mean
1	26,28	26,07	26,34	26,23
2	26,33	26,4	26,14	26,29
3	26,82	26,76	26,64	26,74
4	26,35	26,52	26,32	26,396
	18S rRNA			mean
1	9,82	10,11	9,86	9,93
2	9,88	10,03	10,25	10,053
3	10,15	10,31	10,3	10,253
4	10,34	10,18	10,2	10,24
			mean	10,119
			Sdv	0,1556

Osteoblasts without Strontium				
	Alkaline Phosphatase			mean
1	30,65	30,01	29,78	30,146
2	29,96	29,93	29,69	29,86
3	29,94	30,03	29,39	29,786
4	29,42	29,24	28,94	29,2
	Osteocalcin			mean
1	26,63	26,67	26,6	26,633
2	26,61	26,63	26,6	26,613
3	26,29	26,38	26,45	26,373
4	26,68	26,65	26,4	26,576
	18S rRNA			mean
1	9,72	9,8	9,79	9,77
2	10,38	10,52	10,37	10,423
3	9,93	9,7	9,8	9,81
4	10,02	10,24	10,04	10,1
			mean	10,025
			Sdv	0,303

Gene	Reaction Effici.	Expres.	Std. Error	95% C.I.	**P(H1)**	Result
18S rRNA	0,5	1,000	0,865 - 1,137	0,816 - 1,300	**1,000**	
ALP	0,5	1,268	0,969 - 1,629	0,727 - 2,074	**0,012**	UP
OCN	0,5	1,097	0,980 - 1,197	0,884 - 1,303	**0,049**	UP

This change of gene expression was found to be statistically significant for ALP (p=0.012) and OCN (p=0.049). At the same time, gene expression of the housekeeping gene, 18S ribosomal RNA, which was used as a standard (control) gene for comparison purposes, was not changed (p=1.0) *(Fig. 18)*.

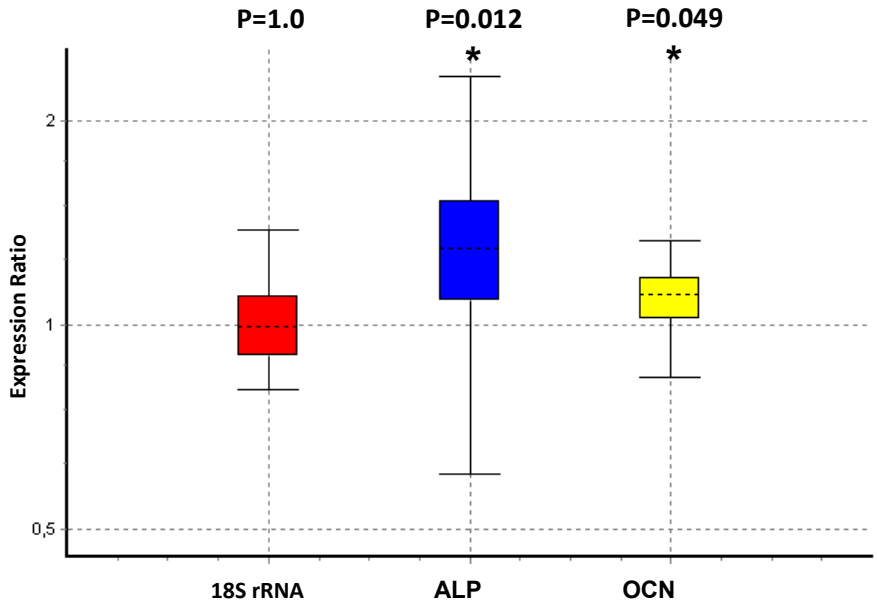


Fig.18. The boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers indicate the minimum and maximum observations. The red, blue and yellow boxes represent 18S rRNA, ALP & OCN gene expression respectively with significant gene up-regulation for ALP and OCN

5.2. Measurement of ALP Activity

Moreover, we analyzed the phenotype of confluent Runx2$^{+/-}$ osteoblasts in presence of 3 mM Sr^{2+} in order to counter-check the validity of previously obtained gene expression data (Section 5.1). For that reason, we determined the volume activity of ALP normalized to the cell number. We found in 3 mM Sr^{2+} exposed Runx2$^{+/-}$ osteoblasts a statistically significant *1.3 fold* increase of ALP activity to 20.4 ± 2nmol/min x ml x 100,000 cells in comparison to osteoblasts in non-Strontium, 0 mM Sr^{2+}, containing osteoblast growth medium (control group) (p=0.02) *(Fig. 19)*.

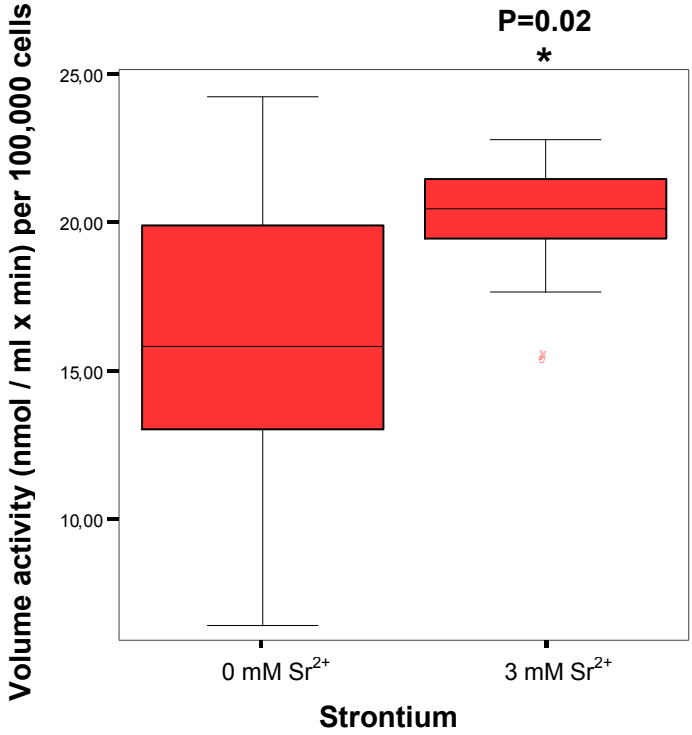


Fig.19. Significant up-regulation of ALP in a certain volume in 3 mM Sr^{2+} treated osteoblasts compared to 0 mM Sr^{2+} treated osteoblasts

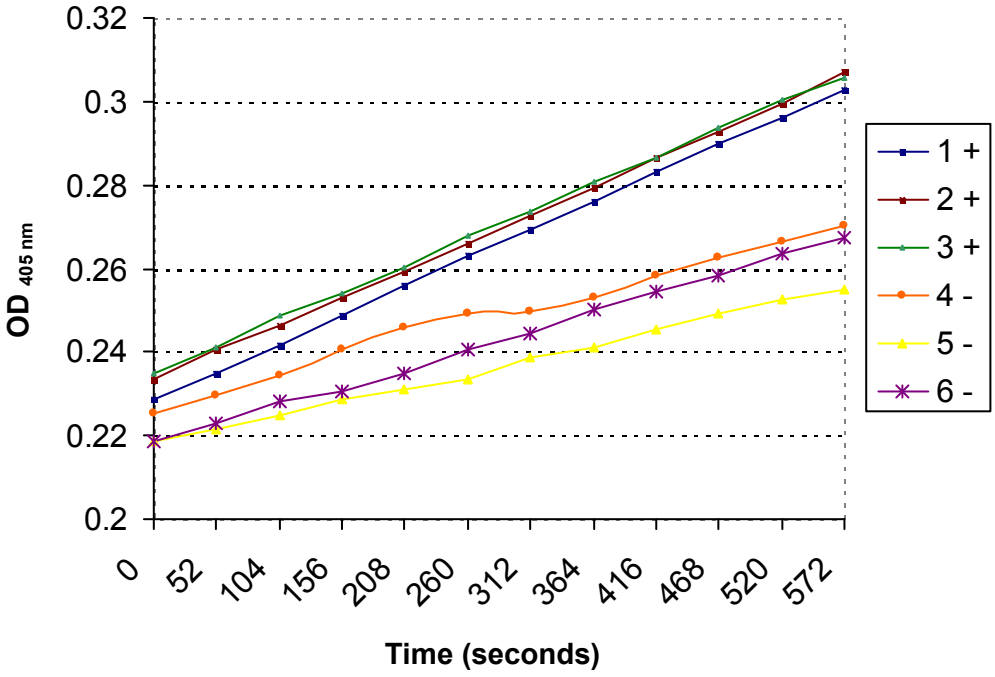


Fig.20. Kinetics of ALP reaction in 96-well plate assay. Note that the first three curves represent the osteoblast groups treated with Strontium ranelate (+). These groups are marked with the colors: green, red and blue. The other three curves marked with orange, purple and yellow represent the osteoblast control groups (-)

5.3. Estimation of Biomineralization Activity

In addition to the ability of Strontium to improve osteoblastic genes expression, it can also increase osteoblast replication, osteoblast differentiation and bone matrix synthesis and mineralization [89, 90, 93, 103].

We estimated the ability of strontium to improve the biomineralization of confluent Runx2$^{+/-}$ osteoblasts in presence or absence of 3 mM Sr^{2+}. Dyeing of extracellular matrix with Alizarin-Red S revealed a statistically significant improvement of the mineralization of Strontium-exposed Runx2$^{+/-}$ osteoblasts in comparison to the Strontium-excluded cell culture *(Fig. 21)*, which validates the results of Real time PCR.

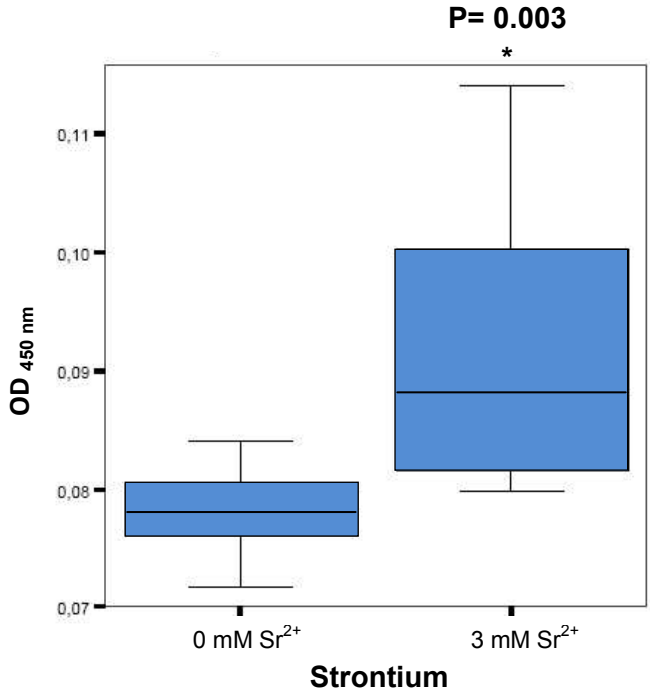


Fig.21. Effect of 3mM Sr^{2+} presence on biomineralization activity. Alizarin-Red S dye was used to measure the bio-mineralization rate in the extracellular matrix. The test demonstrated a significant improvement in the extracellular bio-mineralization in the presence of 3mM Sr^{2+} comparing to the 0 mM Sr2+ control group

5.4. Effect of $SrCl_2$ on Cell Proliferation Rate

Runx2 has a critical role in osteoblast differentiation, function and, consequently, in bone biology [31]. Since Sr^{2+} has a stimulating effect on osteoblast differentiation and maturation, the effect of Strontium in $Runx2^{+/-}$ osteoblasts was tested.

The increase of cell number corresponds with the conversion of WST-1 to formazane, which is detectable at 450 nm *(Fig.22)*. The observed 1.3 fold accelerated growth of $Runx2^{+/-}$ osteoblasts in presence of 3 mM Sr^{2+} from the 1st day up to the 3rd day demonstrates the growth supporting effect of Strontium on the exposed cells as compared to the 0 mM Sr^{2+} control group.

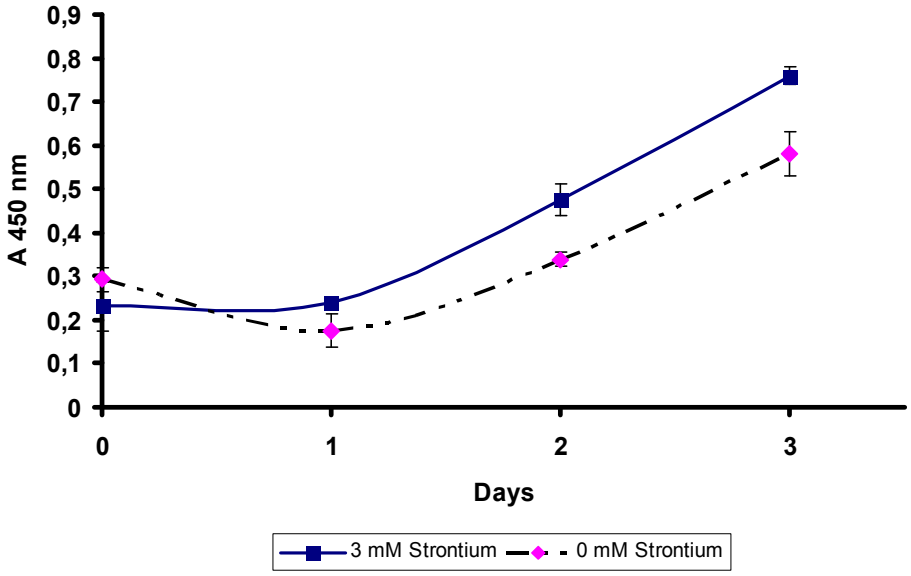


Fig.22. Effect of 3mM Sr^{2+} presence on the cell proliferation rate. Formazane formation was detected at 450nm and used to reflect the cells proliferation process. Proliferation was measured for three days and the results demonstrated improvement in the cell proliferation rate in presence of 3mM Sr^{2+} as compared to the 0 mM Sr^{2+} control group

6. DISCUSSION

Runx2 is considered to be the master transcription factor and the key gene of bone tissue. It plays a major role in all phases of bone formation and is responsible for the initial turnover of pluripotent mesenchymal stem cells into osteoblasts [29]. As well, its presence controls the proliferation, differentiation, and maintenance of these cells [16, 30, 31]. In the dental area, *Runx2* is very essential to the development of calcified tooth tissue, the proliferation of the dental lamina and the maintenance of tooth formation in the later stages [31-33]. Furthermore, *Runx2* regulates the alveolar remodeling process, which is essential for tooth eruption and may play a role in the maintenance of the periodontal ligament integrity [29].

Ectopic expression of *Runx2* in mesenchymal cell lines leads to up-regulation of various osteoblast-related genes like osteocalcin, alkaline phosphatase, collagenase-3, bone sialoprotein and collagen type I [30]. This effect enhances the role of *Runx2* in bone formation and remodeling.

It was shown that homozygous mice with a mutated *Runx2* gene have a complete lack of ossification and both intramembranous and endochondral ossifications are totally blocked due to the maturational arrest of osteoblasts [35] which could result in newborn lethality [22]. As a result, *Runx2*-knockout mice display an absence of osteoblasts and bone formation, while the heterozygous mice (loss-of-function mutations) show specific skeletal abnormalities that are very similar to and characteristic of the human heritable skeletal disorder, Cleidocranial Dysplasia (CCD) [36]. Lou et al. (2009) clearly proved using genetically engineered mouse models that a critical level of Runx2 functional activity is necessary to maintain normal osteoblast differentiation and an adequate bone formation process [49]. However, tissue-specific over-expression of *Runx2* in transgenic mice leads to osteopenia, decreased bone mineral density and multiple fractures and bony defects [52]. These findings were very sensitive and demonstrated the importance of regulation of *Runx2* expression and its activity in bone pathology and biology. They also highlight the fact that any mutations in the *Runx2* gene (heterozygous or homozygous) may cause impairment and a wide range of defects in both the biological and medical level.

It has been hypothesized, that successful activation of Runx2 would lead to improvement of osteoblast differentiation and proliferation, upregulation of osteoblasts-related genes expression and promote ossification in bone formation. As a result, phenotypical symptoms of Cleidocranial Dysplasia may be prevented or at least minimized.

Because of the well-known beneficial effects of Strontium on bone formation and remodeling, we decided to examine the effect of Strontium on primary $Runx2^{+/-}$ osteoblasts which were extracted from a Cleidocranial Dysplasia patient affected with a heterozygous mutation in the *Runx2* gene.

In this study, we used Strontium chloride ($SrCl_2$) instead of Strontium ranelate, because it exhibits very similar effects and properties in animal trials and can be easy dissolved in water which makes its laboratory use and application more reliable [136, 137].

For investigating the effect of Strontium on Runx2+/- osteoblasts we decided to examine gene expression of two characteristic osteoblast-related genes, alkaline phosphatase and osteocalcin. Both gene products are involved in bone tissue formation and mineralization. ALP is highly essential for bone mineralization through hydrolyzing pyrophosphate, which antagonizes the formation of hydroxyl-apatite [138], whereas OCN which is produced exclusively by osteoblasts is believed to play a promoting role in nucleation of hydroxyapatite crystals [139].

A statistically significant up-regulation of osteoblast marker genes, ALP and OCN was recorded. This result was positively confirmed by the Alizarin Red S staining test of the mineralization rate in the extracellular matrix. Improvement in the mineralization of the extracellular matrix of Strontium-exposed $Runx2^{+/-}$ osteoblasts was demonstrated. Moreover, determination of enzyme activity of ALP in the Strontium-exposed $Runx^{2+/-}$ osteoblasts revealed significantly more activity than in cells grown without Strontium. This raise in ALP expression was time-related, which means ALP expression increased with time. Thus, the constant and continuous stimulation effect of Sr^{2+} on the osteoblasts was confirmed.

Similar observations of improved mineralization of extracellular matrix and ALP activity were previously made in Strontium-exposed mesenchymal cells or in pre-osteoblasts from rodent origin. Strontium administration has also shown in vivo

positive results regarding bone formation and enhanced remodeling. Previous studies reported that the exposure of osteoblasts to strontium results in an up-regulation of *Runx2* gene expression [140]. However, in this experiment, we observed no statistically significant change of gene expression of *Runx2* in the primary cell culture (data not shown). In fact, significant up-regulation of gene expression was limited to ALP and OCN without any considerable change of *Runx2* expression.

These findings may be explained by the promoting effect of *Runx2* in the differentiation of pluripotent mesenchymal stem cells into osteoblasts, and its inhibiting role in their maturation. It has been previously noticed that pre-osteoblasts express *Runx2* abundantly in the early stages of cells differentiation. Later, however, it will be down-regulated by immature osteoblasts. In mature cells *Runx2* exerts an inhibiting effect on terminal differentiation, and therefore, is not expressed anymore by these cells [57], which may explain the insignificant improvement in *Runx2* expression in this study.

In 2007, a similar experiment by Zhu LL et al. was done on mesenchymal stem cells, where an increase in the transcriptional activity and phosphorylation level of *Runx2* gene, but not of osteocalcin [96] was noticed. At the same time, experiments on mature osteoblasts showed only a minimal effect on Runx2 expression, but had a positive effect on osteocalcin expression. It has been hypothesized that this might be due to the major role of *Runx2* in promoting and initiating the MSCs differentiation to osteoblasts, whereas osteocalcin is a negative regulator of the differentiation process and is normally expressed in the late stages of osteoblast maturation [96, 124]. We assume that the observed up-regulation of alkaline phosphatase and osteocalcin genes might be due to the promoting effect of the transcription factor Runx2 in the expression of both tested osteoblast-related genes [141, 142] and result from allosterical activation of already existing *Runx2* protein, but not a positive effect of *Runx2* expression.

With respect to structure, *Runx2* has a C-terminal proline/serine/threonine-rich region [46]. This domain mediates ERK/MAPK1 responsiveness, whereas phosphorylation of specific serine-residues leads to enhanced transcriptional activity of *Runx2* [127, 143]. Moreover, it has been demonstrated that Strontium activates the phosphorylation activity of the MAPK signaling pathway, which in turn

promotes the transcriptional activity of *Runx2* in human mesenchymal stem cells [126]. Similar effects of allosterically activation of *Runx2* by MAPK were also reported in human embryonic kidney (HEK293) cells and in murine calvaria osteoblasts treated with Strontium [121, 144]. The osteoblastic growth-supporting effect of Strontium to *Runx*$^{2+/-}$ osteoblasts was clearly evidenced in this investigation. Similar findings suggesting that Strontium preferentially stimulates cell replication in cells of osteoblastic lineage have been reported [93].

Thus far, the mechanism of how Strontium stimulates cell replication and the corresponding signaling pathways is little known and subject of further basic research [145].

7. SUMMARY

This trial provides valuable impulses and information for pharmacological and clinical research aiming to treat, or at least decrease the severity of Cleidocranial Dysplasia symptoms. Until now, Strontium ranelate was exclusively recognized for treatment of osteoporosis in postmenopausal women or for osteoblast growth stimulation for cell biological laboratory purposes. However, we assume, on the basis of this first pilot study and the significant improvement in *Runx*$^{2+/-}$ osteoblast-related genes (ALP and OC), cell proliferation rate and the rise of mineralization in extracellular matrix, that administration of Strontium during embryogenesis and postnatal development by Cleidocranial Dysplasia patients would significantly improve bone formation of the clavicles, accelerate the closure time of fontanels in children and decrease the hazard of bone deformations.

At the same time, we are still aware of potential risks of Strontium due to its inhibiting action of osteoclastogenesis and stimulating effect on osteoclast apoptosis which might interfere with the adaptive bone remodeling process during postnatal growth. Therefore, we recommend further investigations on Strontium administration with genetically engineered *Runx2*$^{+/-}$ mice to evaluate the risk-benefit profile of Strontium in the phase of embryogenesis.

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9. APPENDIX

9.1. Index of Figures

* Regensburg University Hospital Archive, Dental Clinic

9.2. Index of Tables

10. Zusammenfassung

10.1 Einleitung

10.1.1 Die Dysostosis cleidocranialis

Das *Dysostosis Cleidocranialis* Syndrom (DCC) ist durch verschiedene Namen, wie Marie Sainton Syndrom, Mutation Dysostose und Cleidocranial Dysplasia bekannt. Als *Dysostosis cleidocranialis* wird eine seltene, autosomal-dominante, generalisierte skelettale Dysplasie, welche sich nicht nur auf der kraniofazialen Region beschränkt bezeichnet [(1)].

DCC wird in allen ethnischen Gruppen beobachtet und zeigt keine geschlechtsspezifische Prädisposition. Eines der charakteristischsten Merkmale der DCC sind die hypoplastisch oder aplastisch angelegten Schlüsselbeine, die eine Hypermobilität der Schultern bewirken [(4, 5)].

Die DCC ist ein hereditäres Fehlbildungssyndrom, das hauptsächlich die desmale Ossifikation des Schädels und die Ossifikation der Schlüsselbeine betrifft. Die Inzidenz wird auf 1 pro 200.000 Lebendgeburten geschätzt [(6)], die Prävalenz liegt bei 1:1.000.000 [(7)]

Das Syndrom weist andere assoziierte Anomalien, wie kraniofaziale Fehlbildungen, Zahnüberzahl (bis 70 Zähne bei einigen Patienten)[(8)], die Anwesenheit von mehreren retinierten oder verlagerten Zähnen und die Persistenz der Milchzähne bei Dentitio tarda sowie die Hypoplasie des Mittelgesichtes auf. Trotz weiterer skelettaler Befunde sind es vor allem die kraniofazialen Merkmale, die eine Diagnose ausschließlich durch den zahnärztlichen Befund ermöglichen [(9)].

Ein anderes typisches Merkmal der *DCC,* ist der verzögerte oder ausbleibende Verschluss der Nähte des Schädels [(10, 11)].

Die genetische Ursache der *Dysostosis DCC* ist eine Mutationen des ***Runx2* Gens** (osteoblast specific transcription factor) [(12)], welches auf dem kurzen Arm des Chromosoms 6 (6p21) lokalisiert ist [(13)]. Das Genprodukt gehört zur Gruppe der Runt-Transkriptionsfaktoren – die an der Transkription spezifischer Gene beteiligt sind. *Runx2* ist unter anderen an der Reifung der mesenchymalen Zellen zu

Osteoblasten beteiligt [14]. *Runx2* spielt auch die Schlüsselrolle in der Osteogenese. Zahlreiche Publikationen belegen, dass *Runx2* ein integraler Bestandteil vieler Prozesse ist, die während den verschiedenen Phasen der Knochenentwicklung und Knochendifferenzierung ablaufen. [15]

Unter anderem reguliert *Runx2* die Zellproliferation und kontrolliert die Differenzierungsprozesse von mesenchymalen Zellen zu Osteoblasten [14, 16, 17]. Die Rolle in der Mineralisierung von Zahnhartsubstanz und der Einfluss auf die Proliferation der Zahnleiste macht *Runx2* zu einem sehr wichtigen Faktor in der Dentino- und Amelogenese [14, 18, 19].

Die unzureichende Gendosis an funktionellem *Runx2* ist der entscheidende Faktor der *DCC*. Dies führt zu einem Mangel bzw. zu einer Hemmung der Differenzierung der mesenchymalen Stammzellen zu Osteoblasten und damit zu den bekannten skelettalen Defekten.

10.1.2. Die Wirkung von Strontium auf den Knochenmetabolismus

Strontium, das als Strontiumranelat (Protelos®) zur Behandlung bzw. Vorbeugung von Osteoporose seit relativ kurzer Zeit eingesetzt wird, verfügt über einen dualen Effekt in Bezug auf Knochenabbau bzw. Knochenaufbauprozessen [21,31,36]. In vitro-Studien an Osteoblasten zeigten, dass Strontium den Calcium-sensing Receptor (CaSR), sowie die Synthese von Prostaglandin E_2 stimuliert, wodurch eine Aktivierung des MAPK-Signalweges erfolgt [31]. Die durch Strontium induzierte Aktivierung des MAPK-Signalweges in Osteoblasten führt zu einer vermehrten Bildung von charakteristischen Knochenmarkerproteinen (z.B. alkalische Phosphatase, Kollagen Typ I, etc.) und damit zu einer verbesserten Mineralisierung der extrazellulären Matrix [31].

<u>Übersicht über die Wirkung von Strontium auf den Knochenmetabolismus:</u>

1. Senkung der Inzidenz von Wirbel- und Hüftknochenfrakturen [22, 23]
2. Beeinflussung des Knochenumbauprozesses durch eine Erhöhung der Knochenbildung und die Verringerung der Knochenresorption [21]
3. Steigende Knochenfestigkeit und Knochenmasse und allgemeine Verbesserung der Qualität der Knochenstruktur [31, 28, 32-35]

4. Stimulierung der Differenzierung von mesenchymalen Zellen zu Osteoblasten und Beschleunigung der Reifung der Osteoblasten [25, 26]
5. Erhöhung der Synthese von Kollagen Typ I und Förderung der Mineralisierung der Knochenmatrix [36]
6. Unterdrückung der Bildung und Funktion von Osteoklasten und Förderung der Apoptose [25]

10.1.3. Ziel der Doktorarbeit

Bisher ist keine Studie über den Einsatz von Strontium an *DCC* – Patienten bekannt. Aufgrund der in der wissenschaftlichen Literatur beschriebenen Wirkung von Strontium, die zur zusätzlichen Aktivierung von Runx2 durch Phosphorylierung führen, wäre es denkbar, dass die Ossifikationsleistung der $Runx2^{+/-}$-Osteoblasten unter der Wirkung von Strontium verbessert werden könnte. Im Rahmen dieser Doktorarbeit wurde deshalb die Wirkung von Strontium an *DCC* Osteoblasten untersucht, um weitergehende Versuche an geeigneten Tiermodellen (z.B. $Runx2^{+/-}$-Mäusen) vorzubereiten.

10.2. Material und Methoden

10.2.1. Zellkultur

Nach dem Erhalt der entsprechender Einwilligung des Patienten mit *Dysostosis cleidocranialis,* wurden *Runx2*$^{+/-}$ Osteoblasten aus menschlichem Alveolarknochen isoliert und angereichert. Die Analyse der genomischen DNA der Patienten zeigte eine Mutation im *Runx2* -Gen und bestätigte die Erkrankung am DCC Syndrom.
Die $Runx2^{+/-}$ Zellen wurden in einem Nährmedium mit 10%igem Rinderserum und 50 µg/ml Ascorbinsäure, bei 37°C mit 5% CO_2 angereichert (Provitro/Deutschland).

10.2.2. Molekularbiologische Arbeitsmethoden

Die Gesamt-RNA wurde mit dem Tri-Reagent extrahiert. Die gewonnene RNA wurde hinsichtlich der Ausbeute und Reinheit mittels eines UV/Vis-Spektralphotometers (PicoDrop) überprüft. 1 µg Gesamt-RNA wurde mittels einer reversen Transkriptase in cDNA umgeschrieben. Die cDNA wurde danach mittels Real-time-PCR und geeigneter PCR-Primer mit einem „Stratagene MX3000P

qPCR-System“ analysiert. Aus den aufgezeichneten Floureszenzschwellenwerten (CT-Werten) wurde nach der von Livak et al. (2001) publizierten ΔΔCT-Methode ein Genexpressionsverhältnis berechnet.

10.2.3. WST-1-Test

Um die Wirkung von Strontium auf *Runx2*$^{+/-}$ Osteoblasten zu beurteilen, wurden diese Zellen in Abwesenheit (Kontrollgruppe) oder in Gegenwart (Testgruppe) von 3 mM Strontiumchlorid ($SrCl_2$) für 10 Tage auf einer 96-Well-Platte, bei einer Konzentration von 2.800 Zellen pro Well in 100 ul Zellkulturmedium (Merck / Deutschland) kultiviert.
Anschließend wurden 10 ul WST-1 (Roche), sowohl dem Test- und Kontrollmedium beigefügt und die Zellen wurden bei 37°C mit 5%igem CO_2 für eine maximale Zeit von 3 Stunden inkubiert. Die zunehmende Absorption (A = 450 nm) der Probe entspricht der zunehmenden Anzahl an vitalen Zellen.
Die Absorption wurde im 1-Stunden-Abstand (0-4 Stunden) mit Hilfe eines Plattenlesegerätes (Mod. GENIOS, Fa.TECAN/Österreich) ermittelt.

10.2.4. Alkalischer Phosphatase Test

500.000 Zellen der Test (3 mM Sr^{2+}, 6 Proben) - und Referenzgruppe (0 mM Sr^{2+}, 6 Proben) wurden in 100 µl eines Zell-Lysepuffers (CelLytic, Sigma-Aldrich) lysiert. Die Zelltrümmer wurden bei 4°C in einer Tischzentrifuge bei 14000 rpm abzentrifugiert. 50 µl von dem Zellüberstand wurden mittels einem alkalischen Phosphatase-Test (ALP) zur Messung der enzymatischen Aktivität (QuantiChrom™ Alkaline Phosphatase Assay Kit , BioAssay Systems-USA) verwendet.

10.2.5. Biomineralisierungsassay nach Gregory et al (1994)

Durch ein Biomineralisierungsassay nach Gregory et al. (1994) wurde die mit 3 mM Strontium behandelte Testgruppe (n=6) mit 10%igem Formaldehyd fixiert und mit 2%iger Alizarinrot-S angefärbt. Dieser Farbstoff bindet spezifisch fest an kalzifizierter extrazellulärer Matrix. Die fixierten und gefärbten Zellmonolayer wurden nach dem Abwaschen mit Wasser durch einen Spatel abgekratzt und der Farbstoff durch Eisessig in Verbindung mit einer 10 minütigen Inkubation bei 85°C

extrahiert. Die Absorption des extrahierten Farbstoffs wurde bei 405 nm gemessen und mit den Absorptionswerten der Kontrollgruppe (0 mM Strontium, n=6) verglichen und statistisch (U-Test) ausgewertet.

10.2.6. Statistische Auswertung

Die Datenanalyse erfolgte mittels (Student's t-Test), wenn es möglich war, ansonsten über den (Wilcoxon Mann Whitney-Test). Die statistische Auswertung wurde mit dem Programm SPSS durchgeführt. Die Wahrscheinlichkeits-Werte wurden von $p \leq 0,05$ als signifikant angesehen.

10.3. Resultate

10.3.1. Genexpressionsanalyse

Die Genexpression der Osteoblasten wurde 1,3 fach für ALP und 1,1 fach für OCN verbessert. Diese Veränderung der Genexpression wurde als statistisch signifikant für ALP (p = 0,012) und OCN (p = 0,049) angemessen. Gleichzeitig, wurde die Genexpression des Housekeeping-Gens (18S rRNA), das als Referenzgen für die Zwecke des Vergleichs verwendet wurde, nicht geändert (p = 1,0). (Abb. 1)

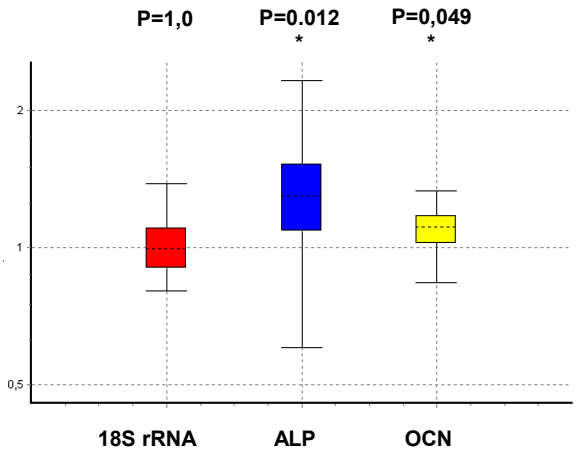

Abb. 1. Genexpression Analyse der Runx2-Gens

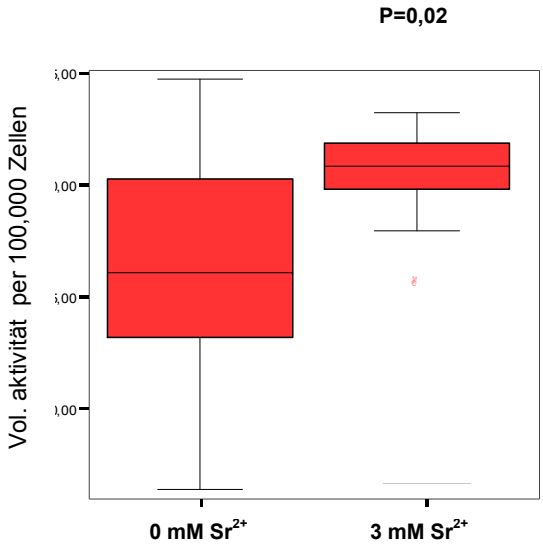

Abb. 2. Messung der ALP Aktivität

Analysiert wurde der Phänotyp von Runx2$^{+/-}$ Osteoblasten in Gegenwart von 3 mM Sr^{2+}. Das Volumen der ALP-Aktivität wurde auf die Anzahl der Zellen normalisiert. Es zeigte sich in 3 mM Sr^{2+} ausgesetzten Runx2$^{+/-}$ Osteoblasten ein statistisch signifikanter 1,3 facher Anstieg der ALP-Aktivität auf 20,4 ± 2nmol/min x ml x 100.000 Zellen im Vergleich zu non-Strontium ausgesetzten Osteoblasten (Kontrolle Gruppe) (p = 0,02) (Abb. 2)

Zusätzlich wurde der Einfluss von Strontium auf die Biomineralisation von konfluenten Runx2 $^{+/-}$ Osteoblasten in Gegenwart oder Abwesenheit von 3 mM Sr^{2+} untersucht. Die Färbung von extrazellulärer Matrix mit Alizarin Red-S zeigte eine statistisch signifikante Verbesserung der Mineralisierung von Strontium

inkubierten Runx2$^{+/-}$ Osteoblasten im Vergleich zur Gruppe der unbehandelten Osteoblasten (Abb.3).

Runx2 hat eine entscheidende Rolle in der Differenzierung und der Funktion der Osteoblasten und damit im Knochenstoffwechsel [31]. Weil Sr^{2+} eine stimulierende Wirkung auf die Differenzierung der Osteoblasten und die Reifung hat, wurde die Wirkung von Strontium auf die Proliferation der Runx2 $^{+/-}$ Osteoblasten getestet.

Die Zunahme der Zellzahl entspricht der Konversion von WST-1 zu Formazan, die nachweisbar bei 450nm ist. Das 1,3 fach beschleunigte Wachstum von Runx2$^{+/-}$ Osteoblasten in Gegenwart von 3 mM Sr2 + ab dem 1. Tag bis zum 3. Tag zeigte aktivierende Wirkung von Strontium auf die Zellproliferation von Runx2$^{+/-}$ Osteoblasten (Abb. 4).

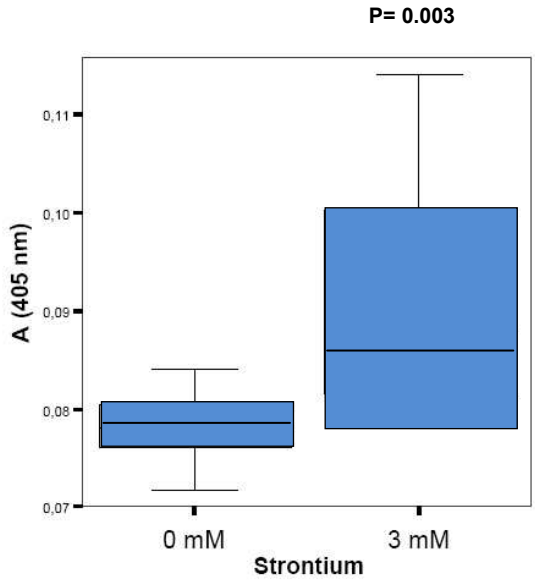

Abb. 3. Biomineralisation Effekt von Strontium auf die Runx2 $^{+/-}$ Osteoblasten

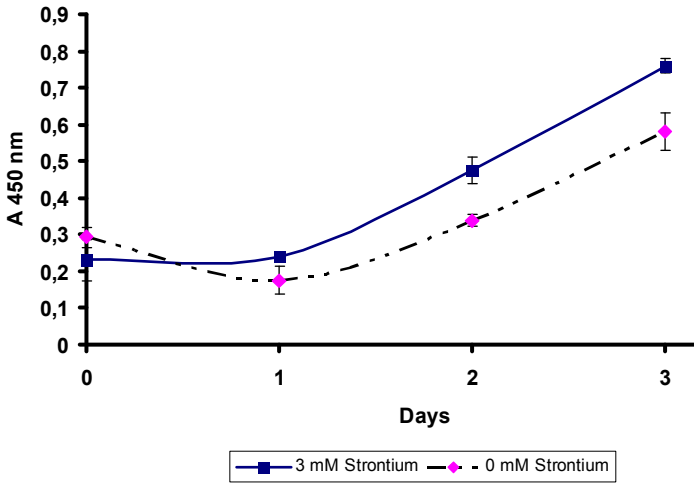

Abb. 4. Wirkung von Strontium auf die Proliferation der Osteoblasten

10.4. Diskussion

Aufgrund der aus der wissenschaftlichen Literatur bekannten positiven Wirkung von Strontium auf die Knochenbildung entwickelte sich die Überlegung, den Einfluss von Strontium auf Runx2$^{+/-}$ Osteoblasten zu untersuchen. Hierfür wurden Osteoblasten von einem Patienten mit dem *Dysostosis cleidocranialis* Syndrom entnommen.

In dieser Studie verwendeten wir Strontiumchlorid ($SrCl_2$) anstelle von Strontiumranelat, aufgrund der guten Löslichkeit des Salzes im verwendeten Zellmedium und der analogen Wirkung, die bereits in *in vitro* und *in vivo-Studien* gezeigt werden konnte [41, 42].
Bei dieser Untersuchung wurde die Genexpression von zwei charakteristischen Osteoblasten-Genen, einerseits die alkalische Phosphatase und andererseits das Osteocalcin, überprüft.

Beide Genprodukte sind im Knochengewebe an der Bildung und Mineralisierung beteiligt. ALP ist essentiell für die Mineralisierung des Knochens durch Hydrolyse von Pyrophosphat [43], während Osteocalcin vermutlich eine Rolle bei der Keimbildung von Hydroxylapatit-Kristallen spielt. [44]
In dieser Studie wurde zwar eine schwache, aber zumindest eine statistisch signifikant verbesserte Transkription der Gene ALP und OCN demonstriert. Dieses Ergebnis wurde durch die Alizarin Rot S Färbung positiv bestätigt, das als Hinweis interpretiert werden kann, dass Strontium die Mineralisierung der extrazellulären Matrix von Runx2$^{+/-}$-Osteoblasten verbessert.

Die Enzymaktivität der ALP in strontiumbehandelten *Runx2*$^{+/-}$ Osteoblasten war signifikant höher als die ALP- Aktivität in unbehandelten Zellen.
Ähnliche Ergebnisse von verbesserter Mineralisierung der extrazellulären Matrix und ALP-Aktivität wurden bei strontiumbehandelten mesenchymalen Zellen oder in Osteoblastvorläuferzellen beobachtet.

Die Ergebnisse dieser ersten Pilotstudie lassen den Schluss zu, dass Strontium zu einer Aktivierung der transkriptionellen Aktivität der Gene für alkalische Phosphatase und Osteocalcin in Runx2$^{+/-}$-Osteoblasten führt, die allerdings im Vergleich zu normalen Osteoblasten sehr schwach ist. Es wird deshalb vermutet, dass die Verbesserung der Biomineralisierungsleistung der Runx2$^{+/-}$-Osteoblasten

vielmehr darauf zurückzuführen ist, dass die Zellproliferation bzw. das Wachstum der Zellen durch Strontium aktiviert wird. Weiterführende Untersuchungen an geeigneten Tiermodellen, wie z.B. Runx2$^{+/-}$-Mäusen sind notwendig, um nachzuweisen, ob die Verabreichung von Strontium an juvenilen Tieren tatsächlich zu einer Verringerung der Symptome von DCC (z. B. verzögerter Suturenschluss, nicht angelegte Schlüsselbeine, etc.) führt.

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